PURIFICATION, CHEMICAL COMPOSITION AND PRIMARY MECHANISM OF ACTION OF AN EXTRACELLULAR ANTIMICROBIAL COMPOUND PRODUCED BY *BEAUVERIA BASSIANA*

By

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DEDICATION

The present work is dedicated to my entire family, especially my father and mother, Mr. and Mrs. B. F. Adefarati, for their parental support and advice and for giving me the gift of life.

To my sisters, and especially my brother, Mr. 'Tunde Adefarati, for their financial contributions toward my education.

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CHAPTER I

INTRODUCTION

Historical Background to the Problem

Research efforts in our laboratory have been directed toward elucidating the mechanism of entomopathogenicity of the hyphomycetous muscardine fungus, Beauveria bassiana. To aid in these research efforts, various ultraviolet-induced mutants were produced (Grula et al., 1978). While screening about 600 soil bacterial isolates by cross-streaking against B. bassiana, the initial observation was made by E. Grula that the growth of four bacilli was inhibited. Further tests showed that one of the bacilli was the most susceptible, and since it was the fourth in a series of tests, it was given the trivial name of Bacillus #4. Since the initial observations, studies have been carried out to purify and determine the mechanism of action and structure of the extracellular agent responsible for the antibiosis.

Bioautography of paper chromatograms of spent media from B. bassiana revealed two zones of inhibition using Bacillus #4 as the test organism. One of the zones occurred at the origin and the other about half-way up the chromatogram (R_f value of about 0.5). The antimicrobial agents corresponding to these zones have been designated origin ('O') and migrating ('M') antibiotics respectively (Champlin, 1981). The present work was done on the migrating antibiotic subsequently

identified as 1-hydroxy-1,2,2-propane tricarboxylic acid (or 2-hydroxy-1,1,2-propane tricarboxylic acid).

Other Antimicrobial Compounds Produced By the Genus *Beauveria*

Members of the genus *Beauveria* are known to produce many biologically active compounds which include insecticidal toxins and antimicrobial compounds. Kobayasi (1977) reported that the Chinese had realized the antibiotic-producting capability of *Beauveria* species over 2,000 years ago. Basyouni et al. (1968) classified two main antimicrobial pigment types produced by *Beauveria* species as red and yellow. The red extracellular pigment has been crystallized earlier, and subsequently identified as oosporein (Vining et al., 1962). The yellow pigments were extracted from the mycelium with hot methanol followed by repeated re-crystallization from the same solvent. Thin layer chromatograms on polymide irrigated with methanol revealed two compounds with R_f values of 0.36 and 0.42. They were both reported as insoluble in water but soluble in ether and were named bassianin and tenellin respectively. Absorption peaks for bassianin were reported as 259 and 378 nm while those for tenellin were 254 and 340 nm. McInnes et al. (1974), in two separate publications, have determined the structure of tenellin and bassianin as 3-acyl derivatives of 1,4-dihydroxy-5-(p-hydroxyphenyl)-2(1H)-pyridone. They further reported that tenellin is derived from acetate, phenylalanine and methionine. Using ^{13}C nuclear magnetic resonance spectroscopy, Leete et al. (1975), deduced that the biosynthesis of tenellin involves an intramolecular rearrangement of phenylalanine.

A class of antibiotics which has been found to be produced by many Beauveria species is cyclodepsipeptides. This group of ionophoric antibiotics act on the cell membrane. Perhaps the single most studied of these cyclodepsipeptides is beauvericin. Hamill et al. (1969) extracted beauvericin from the mycelia of a 76 hr old culture of B. bassiana with methanol and then re-extracted it into ethyl acetate. They determined the structure of beauvericin to be a cyclic repeating sequence of three alternating molecules of N-methyl phenylalanine and three molecules of 2-hydroxyisovaleric acid. It was found to be freely soluble in most organic solvents, and moderately inhibitory to Gram-positive bacteria, fungi, shrimp and mosquito larvae.

Frappier et al. (1975) isolated a cyclodepsipeptide they named beauvellide from a strain of B. tenella. In a 1978 revision, they suggested that beauvellide was actually a mixture of beauverolides H and I or their isomers. They reported that beauverolide H was also independently produced by a strain of B. bassiana. Isogai et al. (1978), isolated a cyclodepsipeptide they named beauverolide A along with beauvericin and bassianolide (an insecticide) from the mycelia of a strain of B. bassiana. Even though Grove and co-workers have reported the isolation of many beauverolides--beauverolides H and I (Grove and Elsworth, 1977); beauverolides A to F, (Grove and Elsworth, 1980); and beauverolides Ba, Ca, Ja and Ka, (Grove, 1980)--information of the detailed structures of these cyclodepsipeptides is lacking at this time.

Early in the course of this study, it was noticed that the 'M' antibiotic produced larger and clearer zones of inhibition when assayed on Sabouraud's Dextrose Agar (pH about 5.6) than on Trypticase Soy Agar (pH about 7.0) using the same assay organism and same concentration of

antibiotic. This prompted an investigation into the effects of pH variation of the antibiotic on its activity. It was found that the antibiotic was more active at acidic pH values than at neutral or alkaline pH values. A search through the literature revealed that similar findings have been reported on other antimicrobial compounds. Sometimes, the effect of acidic pH may just serve to stabilize an antibiotic in aqueous solution. Tanner et al. (1955) reported that anisomycin, an antibiotic produced by two species of Streptomyces, was more stable when stored in aqueous solution at pH 2 than at alkaline pH values. Philip et al. (1956) isolated two antibiotics, ristocetins A and B, from the actinomycete Nocardia laurida. They found that the two antibiotics were stable in acidic aqueous solutions but were inactivated above pH 7.0. An antifungal compound primaricin, isolated from Streptomyces natalensis, was found by Struyk et al. (1957) to be especially stable in aqueous solutions in the pH range of 5 to 7.

Cinoxacin is an antibiotic which is active against most Gram-negative bacteria pathogenic in the human urinary tract. It has been found that the antibacterial activity of cinoxacin against common pathogens of the urinary tract was pH dependent. A four to eight-fold reduction in activity was reported at pH 8 compared with lower pH values (Welling et al., 1982).

The activities of sodium fluoride and iodine, two bactericidal chemicals used in the prevention of tooth decay, have also been found to be pH-dependent. It has been found that for every pH unit decrease between pH 9 and 5, there is a four-fold decrease in the minimum bactericidal concentration (MBC) of sodium fluoride and a two-fold decrease in the MBC of iodine using a broth dilution assay and

Streptococcus mutans as a test organism (Caufield et al., 1982). In the present work, it has been found that the 'M' antibiotic is active at acidic pH values and slightly active at neutral, and alkaline pH values. The activity is enhanced at low pH values with an optimum at about pH 3.0.

How Is the 'M' Antibiotic Different from Other Antimicrobial Compounds Produced by the Genus Beauveria?

Most of the antimicrobial compounds that have been obtained from B. bassiana to date are extractable only from the mycelia. These include beauvericin, tenellin, bassianin and beauverolides. The only extracellular one characterized so far is oosporein, which is a red pigment. Since the antimicrobial compound obtained from the strains of B. bassiana used in the present work is extracellular, could not be extracted from the mycelium and is not red, it was presumed to be different from all others described so far and as such was considered a new one. This was further confirmed by the determination of its chemical composition.

CHAPTER II

MATERIALS AND METHODS

Test Organisms

B. bassiana strain 7 was selected from a previous study from 15 ultraviolet-induced mutants because of its relatively high antibiotic production (Champlin, 1981). Early in the present work, three other strains were tested for antibiotic production and were found to be as good as strain 7. They are B. bassiana strains CC1, CC3, and CC9.

Stock cultures of all B. bassiana strains were grown and maintained on Sabouraud's Dextrose Agar (SDA; Difco Laboratories) slants with 0.3% yeast extract (Difco Laboratories) added to enhance sporulation. Incubation was at 25° C and there was usually good sporulation in 14-16 days after which the strains were transferred to new slants. The old slants were stored at about 4°C; at this temperature, the spores stayed viable for another 7 days for inoculating media used in the experiments (Champlin, 1981).

Assay Organism

Although other bacteria were sometimes included in the various assays, the main organism that was constantly used was Bacillus #4. This organism was isolated from a soil sample by Dr. E. A. Grula and was initially found to be the most sensitive organism to the antibiosis exhibited by B. bassiana S-7 when both were cross-inoculated on SDA

plates (Champlin, 1981). In the present work, however, it has been observed that, of the various organisms tested, Micrococcus lysodeikticus is the most sensitive organism to the 'M' antibiotic. However, Bacillus #4 remained the main test organism for direct comparison with previous work. Based on its morphological and biochemical characteristics, Bacillus #4 could not be assigned a specific name on the basis of the available information in the eigth edition of Bergey's Manual of Determinative Bacteriology (Gibson and Gordon, 1974). The organism has been described fully by Champlin (1981).

Bacillus #4 was grown on Trypticase Soy Agar (TSA; Difco Laboratories) slants at 25°C for 11-12 hrs, then the slants were stored at 4°C for use within 24 hours. Cells from 11-12 hour old slants were used for all assays. Cells from cultures that were allowed to grow more than 12 hrs resulted in clumps rather than a uniform suspension after vortex resuspension in glass distilled water. Sonication for about 5-10 seconds with an ultra-sonic sonifier (Branson Instruments Model LS75) was sufficient to obtain a uniform suspension from slants that had been grown for 12 to 15 hours.

Medium for Antibiotic Production

'M' antibiotic was produced in a chemically defined medium (GAC) containing the following 100 ml total volume (all water used was glass distilled and all components of the medium were ACS grade):

D-glucose	1 g (in 10 ml water)
NH_4Cl	0.1 g
Trisodium citrate	2.58 g (0.1 M)
$MgSO_4 \cdot 7\ H_2O$	3.0 mg

K_2HPO_4	174 mg	20 ml from stock solution
KH_2PO_4	136 mg	

20 ml of trace mineral solution (Grula, 1960) and 47 ml of water were added to bring the total volume to 100 ml. The glucose was autoclaved separately from the rest of the medium at 120°C and 15 psi for 15 minutes. After both components had cooled down to room temperature, the glucose was aseptically added to the rest of the medium. This was done to prevent a brown discoloration of the medium that would have occurred if the glucose had been atuoclaved with the rest of the medium or added when both were still hot. The resulting pH of the complete medium was about 6.85.

Smith and Grula (1981) showed that the three amino acids: L-alanine, L-phenylalanine, and L-valine could meet the minimal carbon, nitrogen and energy requirements of B. bassiana for good germination and growth. When these three amino acids were incorporated into the GAC defined medium, there was preferential production of 'O' antibiotic over 'M' (Champlin, 1981). Since the present work only dealt with 'M' antibiotic, the above three amino acids were excluded throughout.

Fermentation Conditions and Recovery of Spent Medium

A loopful of B. bassiana spores was used to inoculate 100 ml of GAC medium in a 250 ml Erlenmeyer flask. It was stoppered with a cotton plug and incubated at 25°C for 72-96 hours with constant shaking at 120 rpm on a gyrotory shaker (New Brunswick Scientific Company, Model V).

The fermentation medium was centrifuged at about 8,000x g for 10 min for initial clearing. This was then followed by filtration through

a 0.45 μm millipore filter with negative pressure applied.

Disc Antibiotic Assay

Petri plates for disc antibiotic assay were prepared by mixing 12 ml of the appropriate type of agar, usually SDA (56.3 g/l), with 0.35 ml of A_{540}=0.5 suspension of the test organism in glass distilled water. For media other than SDA, the number of grams per liter was decreased proportionally to that of SDA-(56.3 g/l instead of the 65.0 g/l specified on the bottle). This was done to improve the diffusibility of the antibiotic through the softer agar. The agar was kept at about 50°C after steam-melting. It was then poured into a sterile petri plate into which 0.35 ml A_{540}=0.5 of the bacterial suspension had been pipetted. The plate was swirled quickly but gently on a flat bench top for about 10 sec and then left for 10 min to solidify. Whatman #3 MM chromatography paper (13 mm diameter) discs, sterilized by autoclaving, were loaded with the samples to be disc-assayed. A disc to be loaded was picked up with a pair of forceps previously sterilized by dipping it into 95% ethanol and flaming to remove excess ehtanol. Loading was done by touching an edge of the disc to the surface of the sample solution. The solution gradually soaked through the disc and as soon as it reached the opposite edge, the disc was withdrawn and allowed to equilibrate for about 15 sec before placing it on the agar surface. The forceps were re-sterilized for each disc. The plates were then incubated at 25°C for 12-18 hrs. The diameters of zones of inhibition were measured across the discs and this provided an index of the relative concentrations of the antibiotic in the different samples.

Chromatography

Ascending paper chromatography followed by bioautography (sometimes coupled with spraying with color-forming reagents) was used to resolve the antibiotic from impurities otherwise not detectable with the disc assay. Squares of Whatman #1 MM chromatography paper (8" by 8" and 0.16 mm thick) were used. Samples were deposited on a base line 1" from the bottom using a lambda pipette under a stream of cool air blown with a hair dryer. After deposition of samples, the chromatography paper was stapled side to side to form a cylinder. It was then developed in an appropriate solvent system. In the early part of this work, a solvent system consisting of water (65%):isopropanol (22.5%):n-butanol (12.5%) by volume was used (Champlin, 1981).

In studies involving the incorporation of radioactive precursors into the antibiotic, and also chemical composition studies involving the use of spray reagents, two-dimensional chromatography in Heathcote-Jones solvent systems I and II was sometimes used (Heathcote and Jones, 1965).

Chromatograms were developed with 100 ml of the appropriate solvent in 3.8 liter pickle jar until the solvent front was about 1" from the top of the paper. At 25°C, this usually took about 6 1/2 hours. After development was complete, the solvent front was marked with a pencil and the chromatogram was hung to dry in the hood for at least 1 hour at room temperature.

Bioautography

Sterile 22 cm by 34 cm Pyrex plates covered with aluminum foil were used. Two 160 ml medicine bottles, each containing 90 ml of the appropriate melted agar (same concentration as described for the disc

assay) at 50°C were seeded with 6 ml (each with 3 ml) of A_{540}=0.8 of the assay organism. They were then poured onto the sterile Pyrex plate carefully, avoiding splattering and bubbles. The plate was then rocked from side to side quickly before the agar began to solidity (about 20 rocks). The agar was allowed 10 min to solidify. Strips of chromatogram 1 inch wide (0.5 inch on either side of the origin) were then laid on the agar surface. The plate was incubated at 25°C for 12-18 hrs. The strips were removed after the incubation period and a zone of inhibition could be observed wherever there was antibiotic activity. This method was used to confirm the activity of 'M' antibiotic each time an extraction was done.

After centrifugation and filtration, a two-pronged approach was adopted for obtaining the 'M' antibiotic in a relatively pure form. The goals were to work out a batch method of extraction using solvents and at the same time come up with a method of obtaining small quantities of highly purified samples for chemical analysis.

Solvent Extraction

A number of organic solvents were used for extracting the medium. About 1 ml of the spent medium was pipetted into each of two 18 mm test tubes. The pH of the spent medium in one of the two test tubes was lowered from about 7.0 to about 3.0 using a known volume of 1 N HCl. An equal volume of glass distilled water was added to the second tube and each tube was then extracted with 10 ml of some common organic solvent. The organic and aqueous phases were separated and each was tested for antibiotic activity using the disc assay and bioautographic assay to confirm the presence of 'M' antibiotic. Non-volatile or toxic solvents

were either re-extracted with glass distilled water, and the aqueous phase was frozen overnight at -20°C and then lyophilized or was directly air-blown to dryness, redissolved in glass distilled water and then assayed. The original aqueous phase was lyophilized and assayed in the same manner.

Of all the solvents used for extraction, isoamyl alcohol (= isopentyl alcohol = 3-methyl-1-butanol), n-amyl alcohol and isobutyl alcohol phases were found to be active when used to extract spent medium in which the pH had been adjusted to 3.0. However, of the three solvents, isoamyl alcohol extract gave the largest and cleanest zone of inhibition with no tailing or heading on paper chromatograms. None of the solvent extracts were active at neutral pH values.

A precipitate was obtained when acetone was used for extracting the spent medium at a low pH (3.0). Disc assay followed by bioautography showed that there was much more activity in the supernatant than in the precipitate.

It was determined empirically in later experiments that maximum partitioning of the 'M' antibiotic was obtained with acetone by adding 4 to 5 volumes of acetone to 1 volume of the spent medium adjusted to pH 3.0. The supernatant was separated and the acetone was removed from it by evaporating under vacuum in a rotary evaporator (Rotavapor-R, Brinkmann Buchi Type KRvr 65/45) on speed number 2 at about 50°C. The acetone-free supernatant was then frozen overnight at -20°C in 100 ml medicine bottles and lyophilized to dryness. It was stored at -20°C in this dry form until needed.

Further solvent extraction was carried out using isoamyl alcohol. The lyophilized acetone supernatant was dissolved in 1/10th of its

original volume of glass-distilled water to achieve a 10-fold concentration. Ten times the volume of isoamyl alcohol was then added to it in a flask. The flask was vigorously shaken for about 15 min. The isoamyl and aqueous layers were allowed to separate into two layers; each layer was then collected using a separatory funnel. The isoamyl phase was either air-blown dry in the hood and the residue redissolved in 1/10th volume of glass-distilled water and lyophilized, or it was extracted by 1/10th volume of glass-distilled water and lyophilized. The product in either case was a highly hydroscopic yellowish powder. It became oily upon exposure to the air for about 30 minutes. Chromatography followed by bioautography was used to confirm the presence of 'M' antibiotic.

Gel Filtration

A 10 ml sample containing 5 mg/ml acetone supernatant dissolved in glass-distilled water was applied to the top of a Sephadex column with the following characteristics:

Bead Type	Sephadex G-25 (Mesh size = 50 to 150 μm).
Total column height	59.5 cm
Height of packing	51.5 cm
Diameter of column	4.5 cm
Void volume	360 ml
Flow rate	10 drops per minute
Fraction size	2.5 ml (50 drops)
Eluent	Glass-distilled water

The column was run using glass-distilled water as the mobile phase at room temperature and 180 fractions were collected on an automatic fraction collector (Instrumentation Specialties Co., Retriever III.). Each fraction was checked for activity using disc assay. Presence of 'M' antibiotic was confirmed by chromatography followed by bioautography. Active fractions were pooled, lyophilized and stored at -20°C until needed.

Ion Exchange Chromatography

Prior to actual purification of 'M' antibiotic by ion exchange chromatogrpahy, initial experiments were run to determine the pH and the best ionic strength at which the antibiotic would bind to either a cationic or anionic resin. The resins used were carboxymethylethyl Sephadex as the cationic resin and aminoethyl cellulose as the anionic resin (Sigma Chemical Company).

A series of nine 18 mm test tubes were set up for each type of resin, with 0.1 g of the appropriate resin added to each tube. The choice of buffer was made such that the ions do not carry a charge opposite to that of the functional group of the ion exchanger and so would not take part in the ion exchange process. Therefore, Tris (hydroxy-methyl-aminomethane; pK 8.10, pH range 7 to 9) was chosen as the buffer for the anion resin while sodium acetate (pK 4.76, pH range 3.8 to 6.0) was used for the cation resin. The resin in each tube was equilibrated to a different pH by washing ten times with 10 ml of 0.5 M buffer. A pH range of 5 to 9 was used for anion and a range of 4 to 8 for cation exchangers, with 0.5 pH unit intervals between the tubes. The resin in each tube was then equilibrated with buffers at a lower

concentration (0.05 M) to the desired pH by washing five times with 10 ml of the buffer. A 0.1 ml sample of isoamyl alcohol-extracted antibiotic (5 mg/ml) was added to each test tube. The resin was vortexed for about 5 minutes. After settling, the supernatant in each tube was assayed for activity using disc assays. The diameter of the zone of inhibition corresponding to each tube was measured and a decrease in diameter was observed at the pH at which the antibiotic was bound to the resin. This was selected as the binding pH.

The choice of concentration of the starting buffer was made in a similar manner. A set of 10 tubes for each resin was set up as previously described. After equilibration of the resin in each tube with 0.5 M buffer at the selected starting pH (10 X 10 ml washes), the gel in each tube was equilibrated to a different ionic strength using a range of 0.05 to 0.5 M. The resin in each tube was washed five times with 10 ml of the appropriate ionic strength of NaCl with intervals of 0.05 M between tubes at the selected constant pH for each resin. The sample was added and the supernatiant was assayed by disc assay as before. The diameter of the zone of inhibition corresponding to each tube was measured and a decrease in diameter was observed at the ionic strength at which the antibiotic started to bind. The highest ionic strength which permitted binding of the antibiotic and the lowest concentration at which there was no binding were chosen as the starting and eluting ionic strengths for resin.

Having chosen the appropriate ion exchangers, starting buffers and starting and eluting concentrations, 2.5 g of each resin type were swollen in boiled glass-distilled water at 25^{o}C for about 36 hours. Each was then poured into a 20 ml glass syringe barrel from which the

plunger was removed. These then served as the columns. Each column was equilibrated with the chosen starting buffer at the chosen ionic strength by running the buffer through the column until the pH of the eluent was the same as the pH of the buffer as determined with pH indicator paper. A 0.1 ml sample of 10 mg/ml isoamyl alcohol extract was applied to each column. Each column was then washed with about 20 void volumes of the starting buffer. The eluting buffer was passed through each column and forty 1 ml fractions were collected from each on an automatic fraction collector (previously described). Each fraction was disc-assayed for activity. Active fractions were pooled and 'M' antibiotic activity was confirmed by chromatography followed by bioautography. The pooled fractions were lyophilized and stored at -20°C until needed.

Elution from Paper Chromatograms

For spectrophotometric analyses, samples were obtained from paper chromatograms. A 5 mg/ml solution of isoamyl alcohol extract was made in glass-distilled water. Depositions of 30 µl of this solution were made on 8" by 8" Whatman #1 MM chromatography paper. The chromatogram was run in Heathcote-Jones solvent system I. The position of the 'M' antibiotic on the chromatogram was determined based on the R_f value obtained by bioautography. The area around the position of the antibiotic on each chromatographic lane was cut 1 cm on either side. This was then eluted together along with others, in bundles of ten, each with about 5 ml of glass-distilled water at 4°C. One hundred such lanes were cut and eluted. The eluates were pooled and lyophilized. This sample was yellowish and very hygroscopic. Its activity was checked

with the disc assay. It was then stored at -20°C until needed for spectrophotometric analyses.

Acid Activation of the 'M' Antibiotic

As already mentioned in the introduction, it was noted early in the present study that zones of inhibition obtained on Sabouraud's Dextrose Agar (SDA; pH 5.6) were always larger and clearer than those obtained on Trypticase Soy Agar (pH aobut 7.0) with the same assay organism and same concentration of antibiotic. This was investigated further using a working hypothesis that the zones were larger and clearer on the SDA because of its lower pH. The Sephadex column-purified antibiotic was used in experiments designed to probe this phenomenon.

Three experimental tubes and two control tubes were set up as follows: each experimental tube had 0.5 ml of 200 mg/ml 'M' antibiotic at a pH of about 7.0 at the beginning. By adding different amounts of 1 N HCl, the pH of the antibiotic solution in each tube was reduced to a different vlaue, i.e., 2.5, 3.5, and 4.5. All tubes were then brought to the same concentration by adding glass-distilled water to those that received less HCl so that the final concentration in each tube was about 125 mg/ml. One of the control tubes contained 125 mg/ml 'M' antibiotic at a pH of about 7.0, while the second one had potassium phosphate buffer at a pH of 2.5. The purposes of these experiments were to compare the inhibition zone sizes obtained at different pH values and thus determine if the antibiotic could be acid-activated. Control tubes of potassium phosphate buffer were also assayed to determine if pH alone caused inhibition.

The contents of each tube was disc-assayed against eight different organisms: three Gram-positive bacteria-Bacillus #4, Micrococcus lysodeikticus, Staphylococcus aureus; three Gram-negative bacteria - Erwinia carotovora, Escherichia coli, Pseudomonas aeruginosa; and two anaerobic bacteria - Clostridium acetobutylicum and Clostridium butylicum. Incubation of the Bacillus #4 and E. carotovora was at 25°C while the others were incubated at 37°C. After an 18 hr incubation period, the diameter of the zone of inhibition was measured across each disc.

Incorporation of Radioactive Precursors

Champlin (1981) found that there was maximum production of 'M' antibiotic in the presence of citrate when compared with other intermediates of the tricarboxylic acid cycle. It was reasoned, therefore, that citrate may be a direct precursor of the antibiotic. Since a chemically defined medium was used for the production of the antibiotic, radioactive labelling experiments were designed to determine the degree of purification achieved with the acetone-isoamyl alcohol purification protocol previously outlined, to see if the antibiotic would bind to resting or growing cells or both, and to determine which of the three carbon sources; glucose, citrate and aspartate is incorporated best into the 'M' antibiotic.

A 250 ml flask containing 100 ml of GAC medium to which 100 µl of 1,6-^{14}C citrate (trisodium salt) had been added was used. The flask was inoculated with spores of B. bassiana strain 7 and incubated at 25°C for 72 hours. The contents of the flask were then subjected to the acetone-isoamyl alcohol extraction protocol. Small quantities of the acetone

TABLE I

COMPOSITION OF GAC MEDIUM WITH ASPARATE INCLUDED TO SERVE AS ADDITIONAL CARBON SOURCE

D - glucose	1.0 g
Ammonium chloride	0.1 g
Citrate	1.29 g
Aspartate	1.32 g
$MgSO_4 \cdot 7\ H_2O$	3.0 mg
K_2HPO_4	174 mg
KH_2PO_4	136 mg
Trace minerals	20 ml
Glass-distilled water	47 ml
Total	100 ml

precipitate and acetone supernatant were saved. These were chromatographed in duplicate along with the isoamyl extract and 1,6-^{14}C citrate served as the control. One set of chromatograms were exposed to X-ray film (Kodak) and each lane on the other set was cut into one inch strips. Each strip was then immersed into scintillation fluor (Beckman Ready-solv) and counted with a liquid scintillation counter (Beckman, Model 7500).

To determine which of glucose, citrate and aspartate served as the best carbon source for the 'M' antibiotic, the composition of the GAC medium was varied to accomodate aspartate (Table I). Three 250 ml Erlenmeyer flasks were set up each with 100 ml of this medium. Into one flask, 100 µl of 1,6-^{14}C- glucose was added, 100 µl of 1,6-^{14}C-citrate was added to the second flask, and 50 µl of 1,4-^{14}C-asparate was added to the third flask. A fourth flask contained 100 ml of GAC medium to which 100 µl of 1,6-^{14}C- glucose was added. All flasks were inoculated with B. bassiana spores and incubated at 25°C for 72 hours. The spent media were separately subjected to the isoamyl alcohol purification procedure (described previously), and 0.5 ml of each extract was put into scintillation flour and counted as previously specified.

In a separate experiment, Bacillus #4 cells washed off a 12 hr TSA slant with glass-distilled water were treated with isoamyl extract, acetone supernatant, and acetone precipitate obtained from 72 hour GAC spent medium (that contained ^{14}C citrate). In separate test tubes, 0.5 ml of each of the three extracts was added to 9.5 ml A_{540}=0.8 Bacillus #4 cells in distilled water. The cells were filtered and washed with distilled water on a 0.45 m millipore filter under negative pressure.

Radioactivity of each filter was then determined by liquid scintillation counting.

In order to compare the ability of the antibiotic to bind to resting cells with that of growing cells, both types of cells were treated with the isoamyl extract. Growing cells were obtained by growing Bacillus #4 cells in Sabouraud's Dextrose Broth (SDB) to A_{540}=0.8. Resting cells were obtained by washing Bacillus #4 cells grown in SDB with glass-distilled water four times. The cells were then resuspended in water to A_{540}=0.8. In two separate test tubes, 9.5 ml of each type of cell (resting and growing) was treated with 0.5 ml of the isoamyl extract. After allowing 30 min contact time, the cells were washed, filtered and counted with the liquid scintillation counter as before.

Mechanism of Action

In order to determine whether the 'M' antibiotic was bacteriostatic or bacteriolytic, growing and non-growing (also called resting) Bacillus #4 cells were treated with isoamyl alcohol extract of a 72 hour GAC spent medium.

Growing cells were obtained by inoculating 100 ml of Sabouraud's Dextrose Broth (SDB, Difco) with 1 ml of Bacillus #4 cells (A_{540}=0.5) in a 250 ml side-arm Erlenmeyer flask. The Bacillus #4 cells were washed off a 10-12 hr old SDA slant using sterile glass-distilled water. The flask was then incubated at 25°C on a gyrotory shaker with constant shaking at 180 rpm. The growth of the cells in the flask was monitored by measuring the absorbance at 540 nm using a spectrophotometer (Bausch and Lomb, Spectronic 20) at intervals of 1 hour. After about 9 hours,

the cells were in the log phase of growth. At this point, the cells were divided into six sterile and optically clean test tubes, with 10 ml in each tube. Three of these were allowed to continue to incubate in the same medium and under the same conditions. However, before this, 0.5 ml of a 2 mg/ml solution of isoamyl alcohol-purified 'M' antibiotic in water was added to one of the three tubes. A second tube was treated with 0.1 ml of the same solution while a third tube received no treatment and served as a control. The absorbance at 540 nm was measured for each tube before incubation was resumed.

The cells in the other three tubes were washed three times with 10 ml potassium phosphate buffer (pH 5.0); these represented the resting or non-growing cells. The cells in each tube were resuspended in 10 ml of the same buffer followed by treating with the same amount of isoamyl alcohol extract as with growing cells above. The absorbance at 540 nm of each tube was measured and incubation was resumed at 25°C.

The absorbance of each of the six tubes was measured at 30 minute intervals for three hours from the time of treatment with the isoamyl alcohol extract.

This experiment was repeated using M. lysodeikticus instead of Bacillus #4 as the tests organism, since the results obtained with Bacillus #4 could not be conclusively interpreted.

Spectrophotometric Analyses of Chemical Composition

Paper chromatographic eluates of isoamyl alcohol extract was residdolved in water. The sample was first scanned from a wavelength of

800 nm to 200 nm on a Cary 14 spectrophotometer (Applied Physics Corporation) to determine the position of the absorption peak.

It was also subjected to three types of spectrophotometric analyses: ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy, infra-red (IR) spectroscopy and mass spectroscopy (MS).

Nuclear Magnetic Resonance Spectroscopy

^{1}H and ^{13}C NMR were run on a Varian XL-300 NMR spectrophotometer. The sample was dissolved in deuterated TSP ([sodium 3--trimethylsilylpropionate-2,2,3,3-d_4-$(CH_3)_3SiCD_2CD_2CO_2Na$]) (Merck, Sharp and Dome of Canada Ltd). The ^{13}C NMR was continuously proton-decoupled.

Infra-red Spectroscopy

The infra-red spectrophotometer was a Digilab Fourier transform instrument, Model FTS 20-C, operating at nominally $2cm^{-1}$ resolution, but with resolution reduced to about $3cm^{-1}$ by triangular apodization of the interferograms. The spectrum was composed from 100 interferograms co-added in sequence before the Fourier transform was taken. The sample for the infra-red scan was cast as thin film (about 10 μm thick) on a KBr window and mounted on the end of an infra-red gas cell with the sample on the inside. The gas cell was evacuated for about 10 minutes before sampling so moisture and acetone were evacuated from the sample.

Mass Spectrometry

Low resolution mass spectra were obtained on an LKB-9000 combination gas chromatograph/mass spectrometer on the direct probe inlet under the following conditions: 3·5 kV accelerating voltage,

290°C source temperature and 20 μamp trap current. A recording of the total ionization current obtained from the collector plate in the analyzer tube served as the tracing of the emerging compound. Vertical slash marks along the tracings indicate the points where mass spectra were taken. This equipment has been fully described by Waller (1968).

High resolution mass spectra were obtained by fast atom bombardment with a suitable gun and analyzed by Herianna Pang, Department of Chemistry, Massachusetts Institute of Technology, Cambridge, MA. The Varian/MAT 731 equipped with a B-11 N Neutral Atom Gun (i.e., fast atom bombardment gun) manufactured by Ion Tech. Ltd., Headington, England was used as the gas for the gun.

CHAPTER III

RESULTS

Purification of 'M' Antibiotic by Solvent Extraction

The acid form of penicillin has been found to be soluble in organic solvents. This property is utilized in the purification of penicillin in which one of the steps involves extraction into amyl acetate or butyl acetate by a continuous countercurrent process at pH 2.5 to 3.0.

Eight organic solvents--isoamyl alcohol, isobutyl alcohol, n-amyl alcohol, tertiary butyl alcohol, amyl acetate, ethyl acetate, acetone and isopropyl alcohol--were tested in experiments designed to work out a solvent extraction scheme for the antibiotic. It was found that there was general movement of activity from aqueous to organic phases at low pH with all the solvents used (Table II). However, of the solvents used, the most active ones in extracting the antibiotic were found to be isoamyl alcohol and isobutyl alcohol. In the resulting purification scheme, isoamyl alcohol was chosen over isobutyl alcohol; the rationalization being that clearer zones of inhibition were obtained with isoamyl alcohol, even though both gave approximately equal areas of inhibition. All these observations were made using bioautography. The chromatograms were developed in the antibiotic solvent system (water : n-butyl alcohol : isopropyl alcohol in the proportions stated previously), and the test organism was Bacillus #4 grown on SDA.

TABLE II

RELATIVE ACTIVITIES OF EXTRACTS OF GAC SPENT MEDIUM BY SOME ORGANIC SOLVENTS

	pH 6.8	pH 3.0
Isoamyl alcohol	-	++++
Isobutyl alcohol	-	++++
N-amyl alcohol	-	++
Tertiary butyl alcohol	+	++
Amyl acetate	-	++
Ethyl acetate	-	++

Acetone and isopropyl alcohol formed precipitates at both pH values

++++ = Maximum activity

- = No activity

Acetone and isopropyl alcohol caused precipitates to form with the spent medium at both pH values 3.0 and 6.8. While it was noted that a lot more activity was located in the acetone supernatant than the precipitate at low pH, no such clear cut separation was noticeable with isopropyl alcohol precipitation.

Three things became evident at this point which became important in the purification of 'M' antibiotic. These are isoamyl alcohol, acetone, and a low pH. In subsequent experiments, these three factors were combined to obtain a solvent extraction purification scheme. The first step in the purification was to lower the pH of the spent medium to about 3.0 using 1 N hydrochloric acid. It was found by titration that maximum separation was achieved at this pH and also that the antibiotic was found to be most active around this pH (discussed later under acid activation). Initial separation was achieved by adding five volumes of acetone to one volume of spent medium at about pH 3.0.

After separating the supernatant from the precipitate, the acetone was removed from the supernatant by evaporating on a rotary evaporator. The acetone thus recovered was reused to extract another batch of spent medium. The acetone-free supernatant was then concentrated about ten times by lyophilizing and redissolving in one-tenth the volume of glass-distilled water. This solution was then extracted with ten times volume of isoamyl alcohol by re-extracting back into one-tenth the volume of glass-distilled water. The aqueous phase was lyophilized to recover the antibiotic and the isoamyl alcohol could be recycled. The antibiotic could also be recovered from the isoamyl alcohol by air-blowing to dryness, redissolving the residue in water, and then lyophilizing to dryness. This was the preferred method of recovery. (Figure 1

illustrates the whole purification flow chart). At the various stages in the scheme, activities of the various extracts were checked by paper chromatography followed by bioautography using Bacillus #4 grown on SDA (Table III).

Comparison of Gel Filtration with Isoamyl Alcohol Extraction

About 180 ml of acetone-free supernatant was divided equally into three medicine bottles. Each bottle was frozen overnight a -20°C and then lyophilized to dryness. The residue left in one of the bottles was dissolved in 10 ml of glass-distilled water and then extracted with 100 ml of isoamyl alcohol for 30 min with constant shaking in a 250 ml Erlenmeyer flask. The isoamyl phase was separated from the aqueous phase and the latter was then lyophilized to dryness. The isoamyl phase was air-blown dry, redissolved in about 10 ml glass-distilled water and lyophilized to dryness. This yielded a yellowish, oily substance which had been earlier found to have relatively high antibiotic activity. This was redissolved in 10 ml glass-distilled water and washed with 5 ml chloroform which removed the yellow color while full activity was retained.

The contents of the second bottle were dissolved in 10 ml glass-distilled water and run through a column of Sephadex G-25-150 as previously described in Chapter II. The column was eluted with glass-distilled water at room temperature and 180 fractions of 50 drops (about 2.5 ml) each were collected. All fractions that were determined to be active by disc assay were pooled and these included fractions 81 through 140. The pooled fractions were then lyophilized.

Figure 1 Purification of 'M' antibiotic using combined acetone precipitation and isoamyl alcohol partitioning of spent GAC medium.

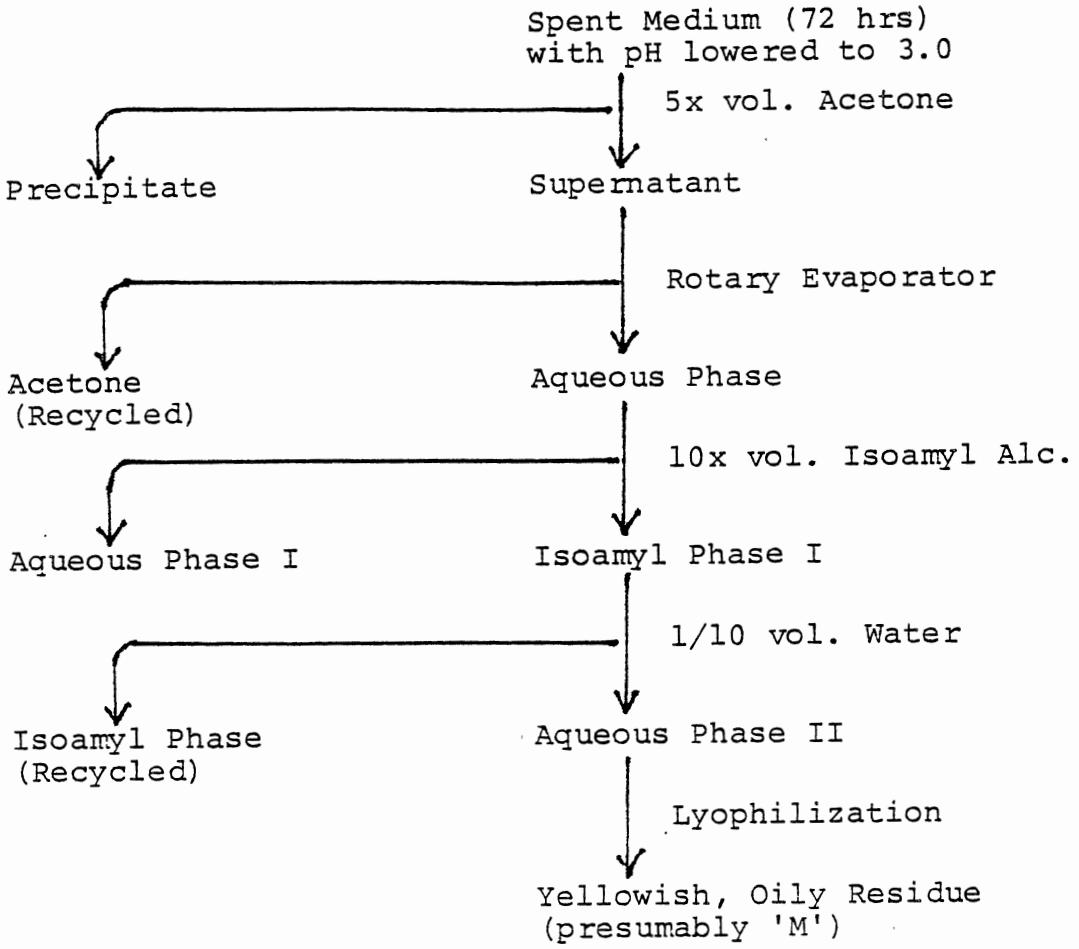

Spent Medium (72 hrs)
with pH lowered to 3.0
5x vol. Acetone
Precipitate
Supernatant
Rotary Evaporator
Acetone
(Recycled)
Aqueous Phase
10x vol. Isoamyl Alc.
Aqueous Phase I
Isoamyl Phase I
1/10 vol. Water
Isoamyl Phase
(Recycled)
Aqueous Phase II
Lyophilization
Yellowish, Oily Residue
(presumably 'M')

TABLE III

QUANTITATION OF BIOAUTOGRAPHIC ASSAYS OF CHROMATOGRAMS FROM DIFFERENT EXTRACTS OF THE ISOAMYL ALCOHOL EXTRACTION SCHEME

	Relative Amounts of Activity
Aqueous phase	+++
1/10 acqueous phase	-
Isoamyl phase	++++++
1/10 Isoamyl phase	+++
Isoamyl alcohol (control)	-
Acetone supernatant	++++++
Acetone precipitate	+

++++++ = Maximum activity

- = No activity

The residue left in the third medicine bottle was not treated further after lyophilization. This served as the control in the comparison of the column purified antibiotic with the isoamyl alcohol extracted antibiotic.

The three samples were compared using disc assays and measuring the diameter of the zones of inhibition. This was followed by bioautography to confirm the presence of 'M' antibitotic. The precipitate obtained from acetone precipitation and the aqueous phase left after isoamyl alcohol extraction were also included as controls.

Solutions of each of the five samples were made to 40 mg/ml, 20 mg/ml and 4 mg/ml in glass-distilled water and were disc-assayed (Table IV). The isoamyl alcohol extract was the most active of all the samples at all three concentrations. Activities of the acetone supernatant, aqueous phase left behind after isoamyl alcohol extraction, and the column-purified sample were roughly the same. The acetone precipitate was only active at a concentraiton of 40 mg/ml and even at this concentration, it was the least active. At a concentration of 4 mg/ml, no activity was detected with the aqueous phase left after isoamyl extraction.

Depositions of 20 µl of the 40 mg/ml samples were made on Whatman #1 MM chromatography paper and run in the antibiotic solvent system. Bioautography of the chromatograms revealed tailing and smaller areas of inhibition on all other samples compared with the isoamyl alcohol extract.

TABLE IV

COMPARISON OF GEL FILTRATION SAMPLE WITH ISOAMYL ALCOHOL EXTRACT USING DISC ASSAY

	Diameter of Zone of Inhibition (mm)		
	40 mg/ml	20 mg/ml	4 mg/ml
Acetone precipitate	16	0	0
Acetone supernatant	24	16	16
Aqueous (after isoamyl)	24	17	0
Isoamyl alcohol extract	29	20	21
Fractions 81-140 of gel filtration	24	16	16

Determination of the Optimum pH and Ionic Strength for Binding 'M' Antibiotic to Cationic and Anionic Ion-Exchange Resins

Table V shows that there is a decrease in the diameter of the zone of inhibition as the pH approaches neutrality from lower values. However, for the anionic resin, there is a relatively large change from 13 mm at pH 7.0 to 9 mm at pH 7.5; and for the cationic resin, from 19 mm at pH 5.5 to 13 mm at pH 6.0. Therefore, the optimum pH for binding the 'M' antibiotic to the anionic resin was taken to be 7.5, while a pH of 6.0 was chosen for the cationic resin.

Table VI shows that for both anionic and cationic resins (especially the anionic resin), the diameter of zone of inhibition was reduced rather sharply at a concentration of 0.25 M sodium chloride. The optimum buffer concentration for binding the 'M' antibiotic was thus chosen as 0.25 M for each resin.

Having chosen the starting buffers, pH values, and buffer concentrations for each resin, the columns were run as described in Chapter II. Elution from the anion exchange column was done using tris-HCl buffer at pH 7.0 and ionic strength of 0.30 M, while sodium acetate buffer at pH 5.5 and ionic strength of 0.30 M was used for eluting the cation exchange column.

Acid Activation

Activation Using Hydrochloric Acid

Four test tubes with 1 ml of 200 mg/ml Sephadex column-purified 'M' antibiotic were set up. The contents of three of the tubes were

TABLE V

DETERMINATION OF OPTIMUM pH FOR BINDING THE 'M' ANTIBIOTIC TO ION-EXCHANGE RESINS

pH	Diameter of Zone of Inhibition (mm)	
	Anion (Tris-HCl Buffer)	Cation (Acetate Buffer)
4.0	-	28
4.5	-	25
5.0	12	21
5.5	13	19
6.0	12	13
6.5	12	12
7.0	13	12
7.5	9	12
8.0	9	12
8.5	9	-
9.0	10	-

TABLE VI

DETERMINATION OF OPTIMUM BUFFER CONCENTRATIONS FOR BINDING THE 'M' ANTIBIOTIC TO ION-EXCHANGE RESINS

Ionic Strength (M NaCl)	Diameter of Zone of Inhibition (mm)	
	Anion	Cation
0.50	12	9
0.45	12	10
0.40	13	11
0.35	13	10
0.30	16	10
0.25	11	9
0.20	11	9
0.15	13	9
0.10	13	9
0.05	11	8

adjusted to pH 2.5, 3.5 and 4.5 using 1 N HCl, while the contents of the fourth tube was left at the initial pH of about 7.0. The final concentration of antibiotic in each of the tubes was adjusted to about 125 mg/ml by adding the appropriate amount of glass-distilled water as necessary. A fifth tube containing potassium phosphate buffer at pH 2.5 (using 1 N HCl) served as a control. The contents of the five tubes were disc-assayed against 8 different organisms and the diameter of the zones of inhibition were measured. The results are tabulated on Table VII. They show a trend of increasing activity as the pH of the medium is reduced from 7.0 to 2.5 with an optimum generally between pH 2.5 and 3.5 for the 8 organisms tested. With all the Gram-negative bacteria (Erwinia carotovora, Escherichia coli, and Pseudomonas aeruginosa) and the Gram-positive Staphylococcus aureus, there was no antibiotic activity at pH 7.0. The antibiotic was quite active against the other Gram-positive organisms which included two aerobes (Micrococcus lysodeikticus and Bacillus #4) and two anaerobes (Clostridium butylicum and C. acetobutylicum). No clear zones were observed with phosphate buffer at pH 2.5 with any of the organisms. A set of disc assays were run on NA plus 0.5% glucose. This was done to see if the test bacteria that are able to produce acid from glucose would be able to activate the antibiotic (at pH 7.0) with the acid they produced. The organisms that can produce acid from glucose are: C. butylicum, C. acetobutylicum, E. carotovora, E. coli and S. aureus. However, only S. aureus produced a larger zone of inhibition on NA plus 0.5% glucose with C. butylicum. There was no activation of the antibiotic by the acid produced by these organisms from glucose. This may be due to the buffering effect of the phosphate buffer which is a constituent of the nutrient agar.

TABLE VII

ACTIVATION OF 'M' ANTIBIOTIC USING HYDROCHLORIC ACID

	DIAMETERS OF ZONES OF INHIBITION (mm)									
	Nutrient Agar					Nutrient Agar + Glucose				
	PB Only	Antibiotic Solution				PB Only	Antibiotic Solution			
pH	2.5	7.0	4.5	3.5	2.5	2.5	7.0	4.5	3.5	2.5
Clostridium butylicum	0	31	31	34	33	0	17	27	31	31
Clostridium acetobutylicum	0	15	23	23	27	0	16	24	27	29
Micrococcus lysodeikticus	0	27	34	38	36	0	29	35	38	38
Bacillus #4	0	15	18	20	20	0	14	20	21	23
Erwinia carotovora	0	0	23	26	24	0	0	20	20	23
Escherichia coli	0	0	22	24	24	0	0	20	21	23
Staphylococcus aureus	0	0	18	18	21	0	0	20	21	29
Pseudomonas aeruginosa	0	0	14	16	17	0	0	15	16	17

Diameter of disc = 13 mm; Contact time of Ab'ic and acid was 30 min at 25oC

PB = Potassium phosphate buffer

Activation Using Lactic Acid

The pH of a 200 mg/ml solution of the 'M' antibiotic was lowered from about 7.0 to 4.0 by adding about the same volume of 50 mM lactic acid. An equal volume of water was added to another tube containing an equal amount of the 'M' antibiotic solution at a pH of about 7.0. This along with a tube of 50 mM lactic acid served as the control tubes. The experimental and control tubes were disc-assayed against S. aureus and M. lysodeikticus on NA. Table VIII shows that the unacidified antibiotic (pH 7.0) and the 50 mM lactic acid gave 30 mm and 20 mm zones of inhibition respectively with M. lysodeikticus. There were no zones of inhibition observed with S. aureus. However, with the lactic acid-activated antibiotic, a zone of 39 mm was obtained with M. lysodeikticus (much larger than any of the zones obtained with the control tubes) and a zone of 21 mm was obtained with S. aureus. This shows that not only hydrochloric acid, but also lactic acid and perhaps other acids can activate the antibiotic, making it more potent.

Stability of Acid-Activated 'M'
Antibiotic Against pH Changes

Once acid activated, does the antibiotic stay activated despite changes in pH? This question was answered with the following experiment. The pH of 0.5 ml of 200 mg/ml of the antibiotic was lowered from 7.0 to 2.5 by adding 4 drops of 4 N HCl from a Pasteur pipette. Four drops of glass-distilled water were also added. The pH of a similar sample was lowered from 7.0 to 2.5 by adding 4 drops of 4 N HCl and then brought back to pH 7.0 by adding 4 drops of 4 N NaOH. Eight drops of glass-distilled water were added to a third tube containing a

TABLE VIII

ACTIVATION OF 'M' ANTIBIOTIC USING LACTIC ACID

	Diameters of Zones of Inhibition (mm)		
	Lactic Acid Alone at pH 2	'M' Antibiotic Alone	'M' Antibiotic + Lactic Acid pH 4
M. lysodeikticus	30	20	39
S. aureus	--	--	21

similar sample. The fourth tube which served as a control contained phosphate buffer with the pH lowered to 2.5 using 4 N HCl. All tubes were disc assayed using S. aureus, E. coli, and M. lysodeikticus grown on NA plus 0.5% glucose as test organisms. The results are shown in Table IX. It shows that the phosphate buffer alone had no activity toward any of the organisms. At pH 7.0, only with M. lysodeikticus, was a 27 mm zone of inhibition obtained. When the pH was lowered to 2.5, zones of inhibition were obtained with all three organisms: S. aureus, 27 mm; E. coli, 23 mm; and M. lysodeikticus, 39 mm. When the pH was first lowered to 2.5 from 7.0 and then raised back to 7.0, the same size of inhibition zones that were obtained with the original antibiotic at pH 7.0 were measured. This shows that the antibiotic is more active at lower pH values.

In all experiments on acid activation in which different bacteria were used as assay organisms, M. lysodeikticus proved to be the most susceptible organism to the antibiotic.

^{14}C-Labeling of 'M' Antibiotic

Champlin (1981) reported that of the various glycolytic and TCA intermediates that were checked for incorporation into the 'M' antibiotic, citrate was the most effective precursor. He further reported that even though the most antibiotic was produced in the presence of citrate as determined by the diameter of inhibition zones on disc assay plates, for growth to take place at all, glucose was the preferred carbon source. Against this background, an experiment was designed to see which of three carbon sources is incorporated best into the 'M' antibiotic as previously described. The three carbon sources

TABLE IX

STABILITY OF 'M' ANTIBIOTIC AGAINST pH CHANGES

	Diameters of Zones of Inhibition (mm)			
	PB*	'M' Antibiotic	'M' Antibiotic	'M' Antibiotic
pH	2.5	7.0	2.5	2.5 7.0
S. aureus	-	-	27	-
E. coli	-	-	23	-
M. lysodeikticus	-	27	39	27

*Potassium phosphate buffer

used were 1,6-^{14}C glucose, 1,6-^{14}C citrate, and 1,4-^{14}C-aspartate. The results (Table X) show that ^{14}C-citrate gave the highest labeling in the antibiotic. It also shows that the ^{14}C citrate is at least 60 times as efficient a precursor as ^{14}C-glucose and more than twice as efficient as ^{14}C-asparate.

After establishing that citrate is the best of the three carbon sources for labeling the antibiotic with ^{14}C, the next two experiments were designed to locate the position of the ^{14}C 'M' antibiotic relative to ^{14}C-citrate and other extracts of the isoamyl alcohol purification protocol on autoradiograms.

The pattern of radioactivity obtained with the different extracts is shown in Figure 2. The 'M' antibiotic (isoamyl alcohol extract) migrated a little farther than citrate in the solvent system. The antibiotic also moved as a compact spot with no heading or tailing. On the contrary, the acetone precipitate appeared as a long smear. The acetone supernatant appeared as a shorter smear but not as a compact spot as the isoamyl extract. The pattern obtained here probably gives some indication of the levels of purification obtained at the various stages of the isoamyl alcohol extraction procedure. It shows that the antimicrobial compound moves close to trisodium citrate in the solvent system used and this may be an indication that its molecular weight is similar to that of citrate.

Scintillation counts of one-inch chromatographic strips of ^{14}C-citrate, isoamyl extract, acetone precipitate and acetone supernatant were plotted as disintegrations per minute (DPM) against distance of the one-inch strip from the origin end of the chromatographic paper in inches. The results are shown in Figure 3 (a to d). The radioactivity

TABLE X

INCORPORATION OF SOME ^{14}C COMPOUNDS INTO 'M' ANTIBIOTIC

Medium	Radioactive Non-radioactive C (M)	Dilution Factor	Count of Isoamyl Alcohol Extract (DPM)	(Dilution Factor) x (Count) (DPM)	Corrected Count/ Relative Activity (DPM)	Relative Efficient of Incorporation
*Glc NH_4Cl Cit	1 : 2.17 x 10^5	1.5	8	12	0.15	1
*Glc Asp Cit	1 : 9.11 x 10^5	6.4	8	51.2	0.63	42
Glc *Asp Cit	1 : 5·96 x 10^6	41.7	6	250.2	15.64	104.3
Glc Asp *Cit	1 : 1.42 x 10^5	1.0	38	38	38	253.3

*Glc = 1.6-^{14}C-glucose · Specific activity = 279 µCi/mM (0·46 mM used)

*Cit = 1.6-^{14}C-citrate · Specific activity = 22.0 µCi/mM (0·072 mM used)

*Asp = 1.4-^{14}C-aspartate · Specific activity = 232 µCi/mM (0·11 mM used)

Relative activity - *Glc : *Cit : *Asp = 1.28 x 10^8 : 1·58 x 10^6 : 2·55 x 10^7
= 81 : 1 : 16

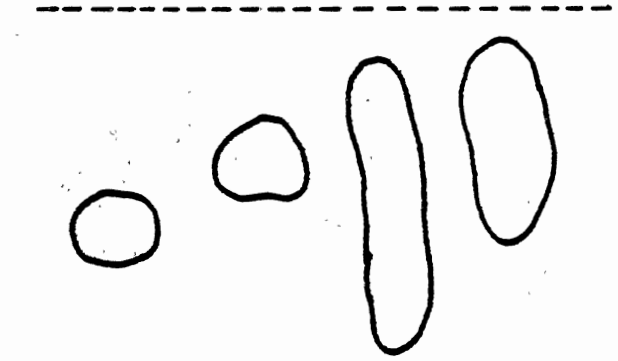
Figure 2. Autoradiograms of different extracts of the isoamyl alcohol purification scheme.

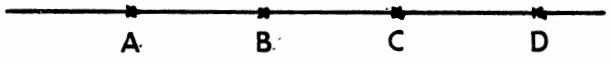
A. Radioactive citrate

B. Radioactive isoamyl alcohol extract

C. Radioactive acetone precipitate

D. Radioactive acetone supernatant




A
B
C
D

obtained for the acetone precipitate was spread over four inches (4 strips). This probably shows that the presence of a wide variety of unresolved compounds in this fraction. It has a peak at six inches and this corresponds with the radioactive citrate peak. However, the acetone supernatant and the isoamyl alcohol extract have their peaks at seven inches which corresponds to the antibiotic.

Labeling of Bacillus #4 Cells with Radioactive Extracts

The Bacillus #4 cells were treated with isoamyl alcohol extract, acetone supernatant, and acetone precipitate in three separate test tubes as described in Chpater II. The liquid scintillation counts obtained with the isoamyl alcohol extract (Table XI) was more than double that obtained for the acetone supernatant (1335 and 633 DPM respectively). Since these are the two fractions that contain the antibiotic, with the isoamyl alcohol extract purer than the acetone supernatant, more binding to cells in pressence of isoamyl extract was observed as expected. A count of 1815 DMP was obtained from cells treated with acetone precipitate. Impressive as this count may be, it has been found in previous experiments that the acetone precipitate contained little or none of the 'M' antibiotic and so the labeling might have been due to some other metabolites other than the 'M' antibiotic.

Table XII shows counts obtained from growing and resting cells separately treated with the radioactive isoamyl alcohol extract. The labeling was done in triplicate for each cell type. No significant difference between growing and resting cells was evident.

Figure 3. Scintillation counts of one-inch strips of the chromatograms of the different extracts obtained from the isoamyl alcohol purification scheme.

a. Radioactive citrate

b. Radioactive isoamyl alcohol extract

c. Radioactive acetone precipitate

d. Radioactive acetone supernatant

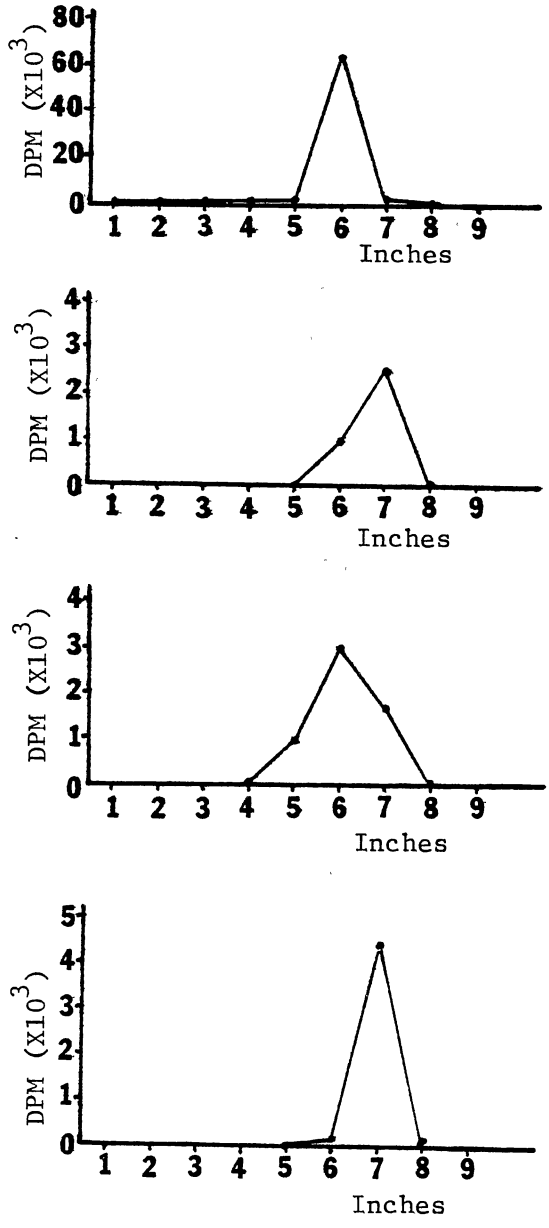

DPM (X10^3)
80
60
40
20
0
1 2 3 4 5 6 7 8 9
Inches
(a)
DPM (X10^3)
4
3
2
1
0
1 2 3 4 5 6 7 8 9
Inches
(b)
DPM (X10^3)
4
3
2
1
0
1 2 3 4 5 6 7 8 9
Inches
(c)
DPM (X10^3)
5
4
3
2
1
0
1 2 3 4 5 6 7 8 9
Inches
(d)

TABLE XI

LABELING OF *BACILLUS* #4 CELLS WITH DIFFERENT EXTRACTS OF ^{14}C-CITRATE LABELED GAC MEDIUM

	DPM
Unlabeled cells	43
Cells labeled with isoamyl alcohol extract	1335
Cells labeled with acetone supernatant	633
Cells labeled with acetone precipitate	1815

TABLE XII

COMPARISON OF GROWING AND RESTING
BACILLUS #4 CELLS LABELED WITH
ISOAMYL ALCOHOL EXTRACT

		DPM	Mean DPM
Growing cells	1	273	
	2	251	257
	3	246	
Resting cells	1	321	
	2	298	332
	3	378	

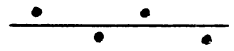
1, 2 and 3 are replicate samples.

Growing Versus Resting Bacillus #4 Cells

Growing Bacillus #4 cells were treated with 5 mg/ml and 1 mg/ml isoamyl alcohol extract while growing in SDB. Control cells were not treated. The isoamyl alcohol extract was applied to the treated cells at about the ninth hour of growth when the cells were in the logarithmic phase of growth. Resting cells obtained from the same phase of growth by washing away the nutrients (SDB) with sterile glass-distilled water were treated in the same manner with the isoamyl alcohol extract. Figure 4 (a) shows that after the growing cells were treated at A_{540}=0.15 with the antibiotic, it took two hours before the absorbance started leveling off at about 0.30 for cells treated with 1 mg/ml and at about 0.33 for cells treated with 5 mg/ml. The control cells started leveling off at 0.43 about 1 hour later. There was no significant difference between the times it took for the cells treated with the two different concentrations of the antibiotic to level off. Also since the control cells started leveling off shortly after the treated cells, no definite conclusions could be drawn.

When the cells were washed to obtain resting cells from SDB-grown cells, the absorbance of the cells in the three test tubes (two experimental, one control) became very different after they were resuspended in sterile glass-distilled water. This was probably caused by the loss of unequal numbers of cells from each test tube during washing. It could still be observed, however, that after treating two of the tubes with 5 mg/ml and 1 mg/ml 'M' antibiotic, the absorbance decreased at about the same rate in both tubes over a period of about 3 hours (Figure 4b). In the control tube, there was an initial slight drop in absorbance in the first 15 min which leveled off at 0.035 for

Figure 4a. Effect of different concentrations of 'M' antibiotic on Bacillus #4 cells growing in Sabouraud Dextrose Broth.



Untreated growing cells (control)

Growing cells treated with 5 mg/ml 'M' antibiotic

Growing cells treated with 1 mg/ml 'M' antibiotic

Arrow shows time of treatment of cells.

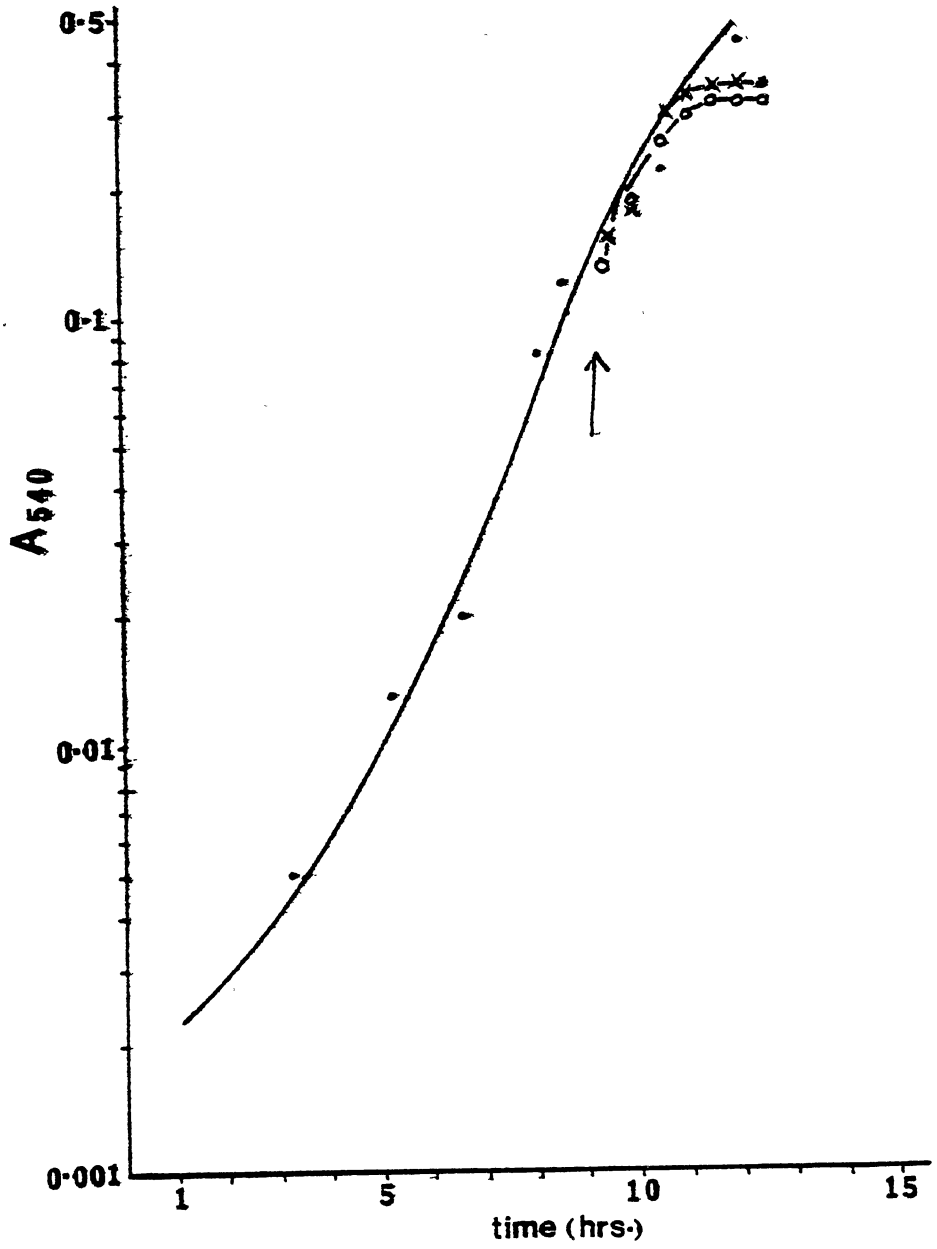
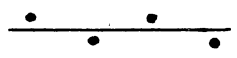

0·5
0·1
0·01
0·001
A_{540}
1
5
10
15
time (hrs·)

Figure 4b. Effect of different concentrations of 'M' antibiotic on resting Bacillus #4 cells.

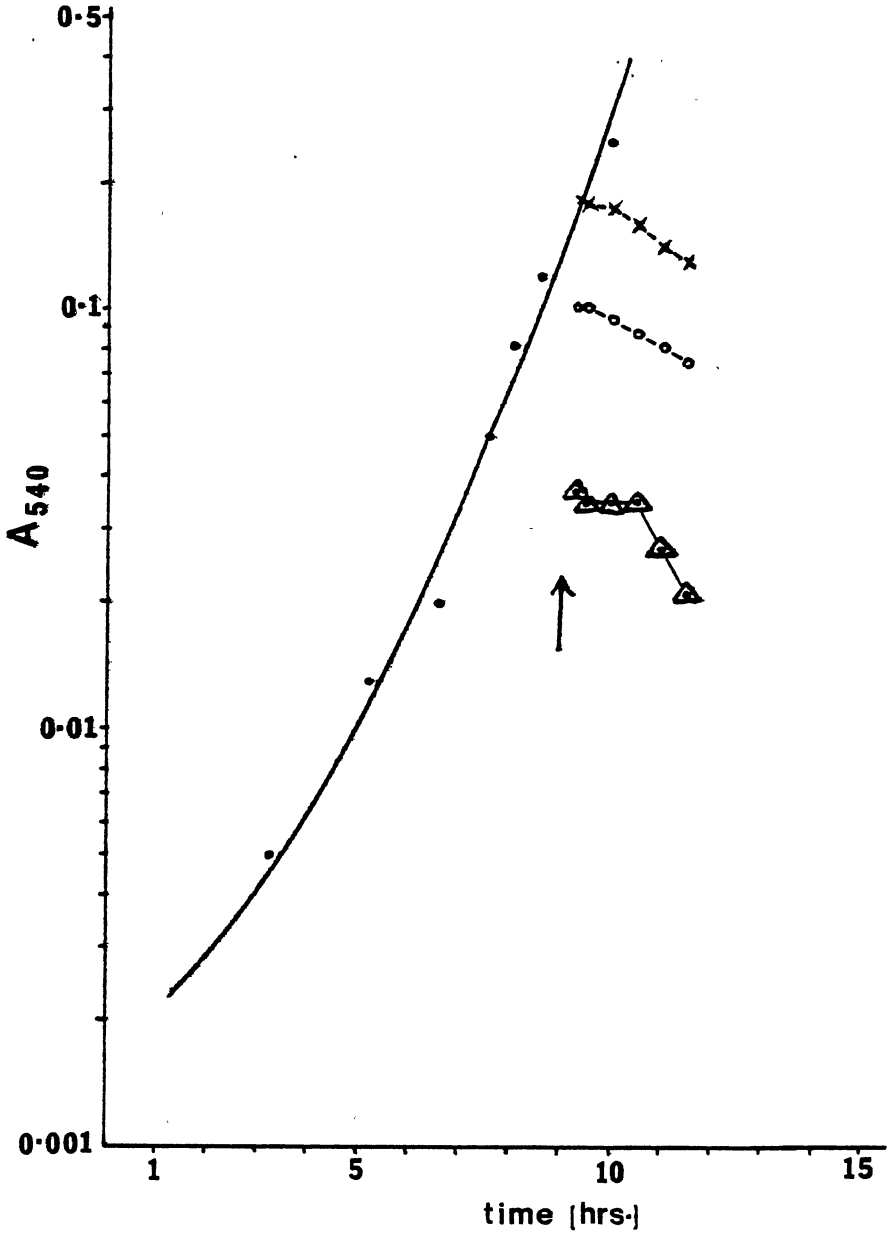

Cells growing in Sabouraud Dextrose Broth (control)


Resting cells treated with 5 mg/ml 'M' antibiotic


Resting cells treated with 1 mg/ml 'M' antibiotic


Resting cells (control)

Arrow shows time of treatment of cells.


0·5
0·1
0·01
0·001
A_{540}
1
5
10
15
time (hrs.)

one hour before finally dropping rather sharply to 0.021 in the next 1 hour. The period when the absorbance leveled off with the control also corresponded with a time when the absorbance of the treated cells was falling. It appears, therefore, that the treatment of the cells with the 'M' antibiotic hastened the decrease in absorbance of the treated cells. The sharp drop in absorbance of the control cells in the last 1 hour might have been due to normal lysis of the cells in a hypotonic medium.

Comparing the growing with non-growing cells, it appears that there was generally a greater decrease in absorbance with the non-growing cells than with the growing cells.

Growing Vs. Resting M. lysodeikticus Cells

Growing and resting M. lysodeikticus cells were obtained in a similar manner to the description for Bacillus #4 except that Tryptic Soy Broth (TSB) was the medium used instead of SDB (M. lysodeikticus does not grow well in SDB). Also the cells were treated with three different concentrations of the antibiotic (0.04, 0.4 and 4 mg/ml). The experiment was set up to clear some doubts and questions that were raised by the Bacillus #4 cells treatment experiment. It has been established in our laboratory that Bacillus #4 has very high autolytic enzyme activity and it was thought that this might have interfered with the results obtained with Bacillus #4. On the other hand, the organism that is usually considered almost totally devoid of autolytic enzyme activity is M. lysodeikticus, and so it was chosen for these experiments.

The growing cells were treated with the different concentrations of the antibiotic in the log phase (10 hours, 20 minutes). Cells treated with 0.04 mg/ml (40 mg/ml) continued to grow but at slightly lower absorbance than the control cells (Figure 5a). Those treated with 0.4 mg/ml and 4 mg/ml started leveling out in absorbance almost as soon as they were treated, but while cells treated with 0.4 mg/ml leveled out at an absorbance of 0.25, 4 hrs after treatment at 0.13, those treated with 4 mg/ml leveled off at an absorbance of 0.16, 2 hours after treatment. Just like the Bacillus #4 cells, the cells of M. lysodeikticus were washed and resuspended in

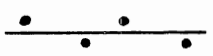
sterile glass-distilled water, the tubes came out at much varied absorbances as shown by an arrow indicating the time of treatment of cells with the 'M' antibiotic in Figure 5b. In this case, however, there was a decrease in absorbance even with 0.04 mg/ml antibiotic concentration. There was no significant difference between the decrease in absorbance obtained at the different concentrations. Cells that were not treated with the antibiotic but were washed free of nutrients also showed a drop in absorbance but the decrease was delayed about 1 hour as against an immediate effect noticed with the treated cells. The control growing cells continued to grow exponentially.

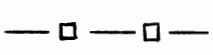
Chemical Composition

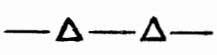
The initial scan of a sample of the 'M' antibiotic on a Cary 14 spectrophotometer revealed an absorption peak in the ultraviolet region at about 275 nm (Figure 6).

The exact molecular weight of the compound was determined using the fast atom bombardment source and a high resolution mass spectrometer.

Figure 5a. Effect of various concentrations of 'M' antibiotic on Micrococcus lysodeikticus cells growing in Sabouraud Dextrose Broth.


Growing cells (control)


Cells treated with 0.04 mg/ml 'M' antibiotic


Cells treated with 0.4 mg/ml "M" antibiotic


Cells treated with 4 mg/ml 'M' antibiotic

Arrow shows time of treatment of cells.

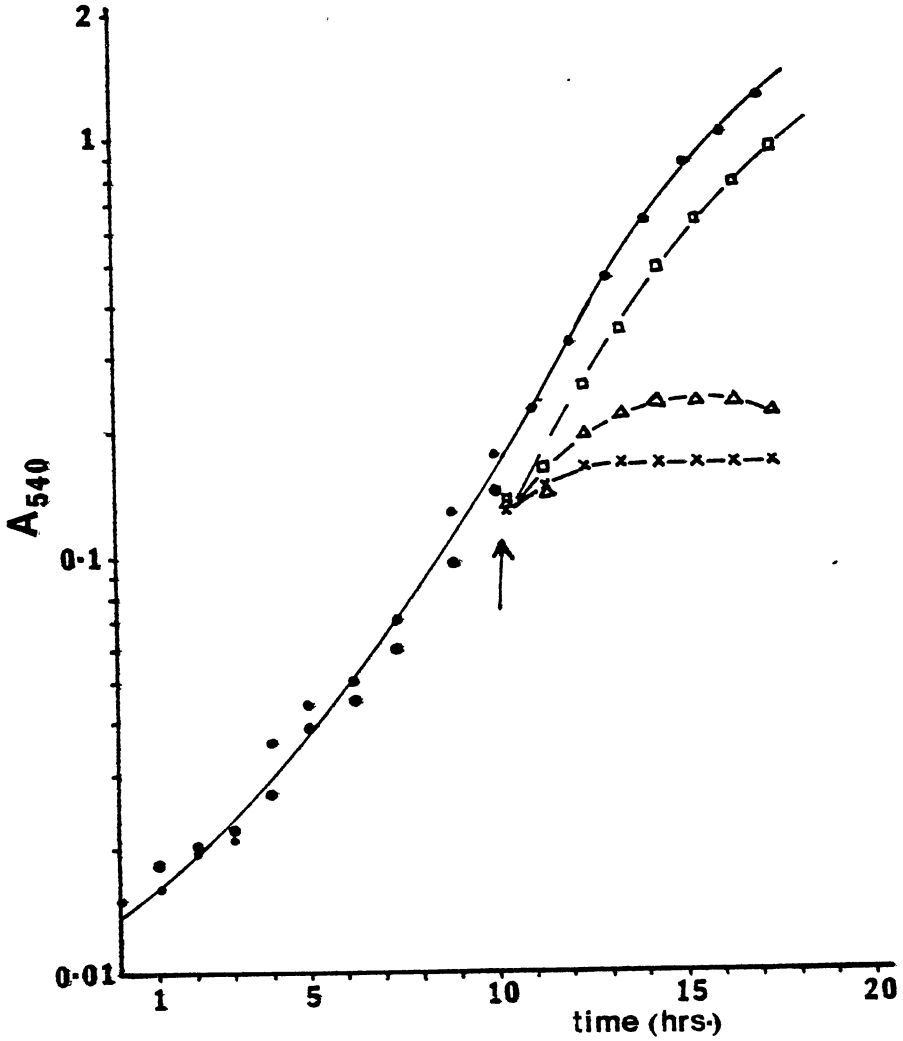
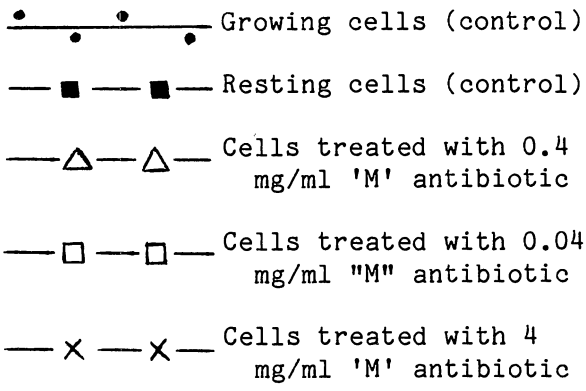

2
1
0·1
0·01
A_{540}
1
5
10
15
20
time (hrs·)

Figure 5b. Effect of various concentrations of 'M' antibiotic on resting Micrococcus lysodeikticus cells.



Arrow shows time of treatment of cells.

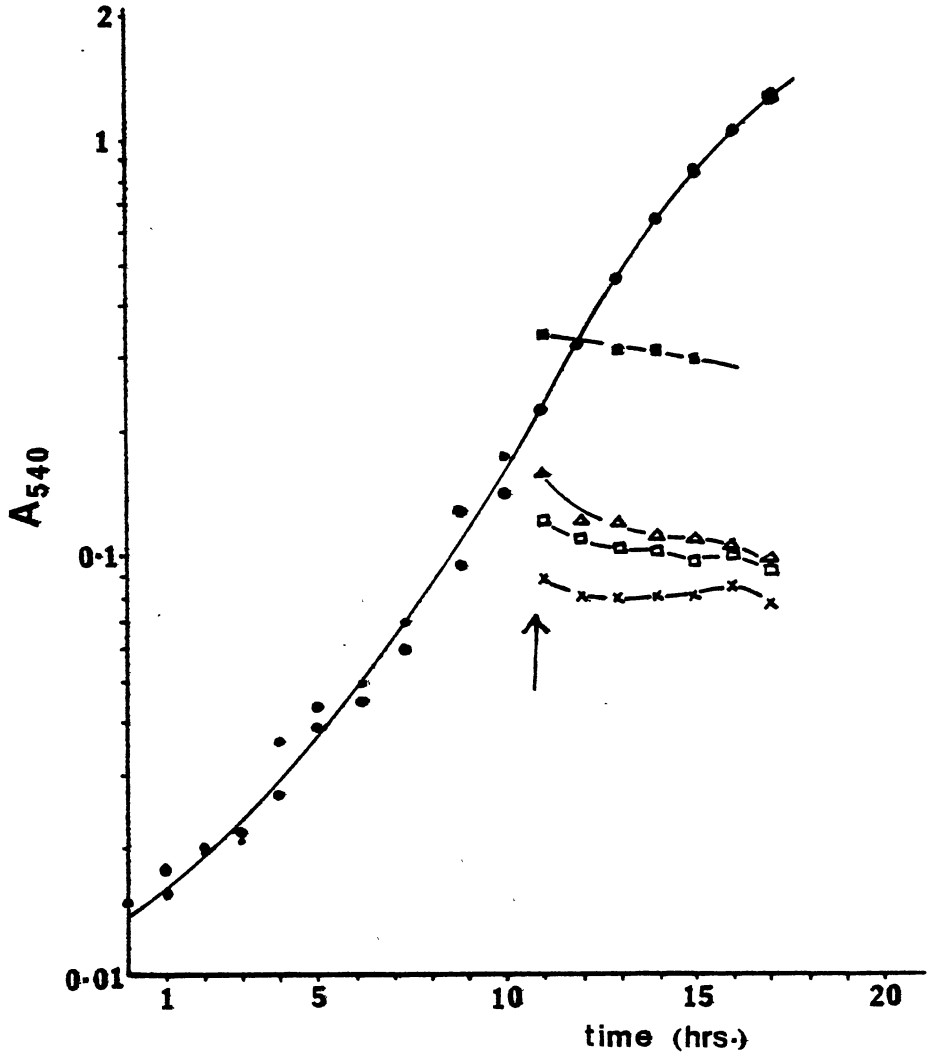
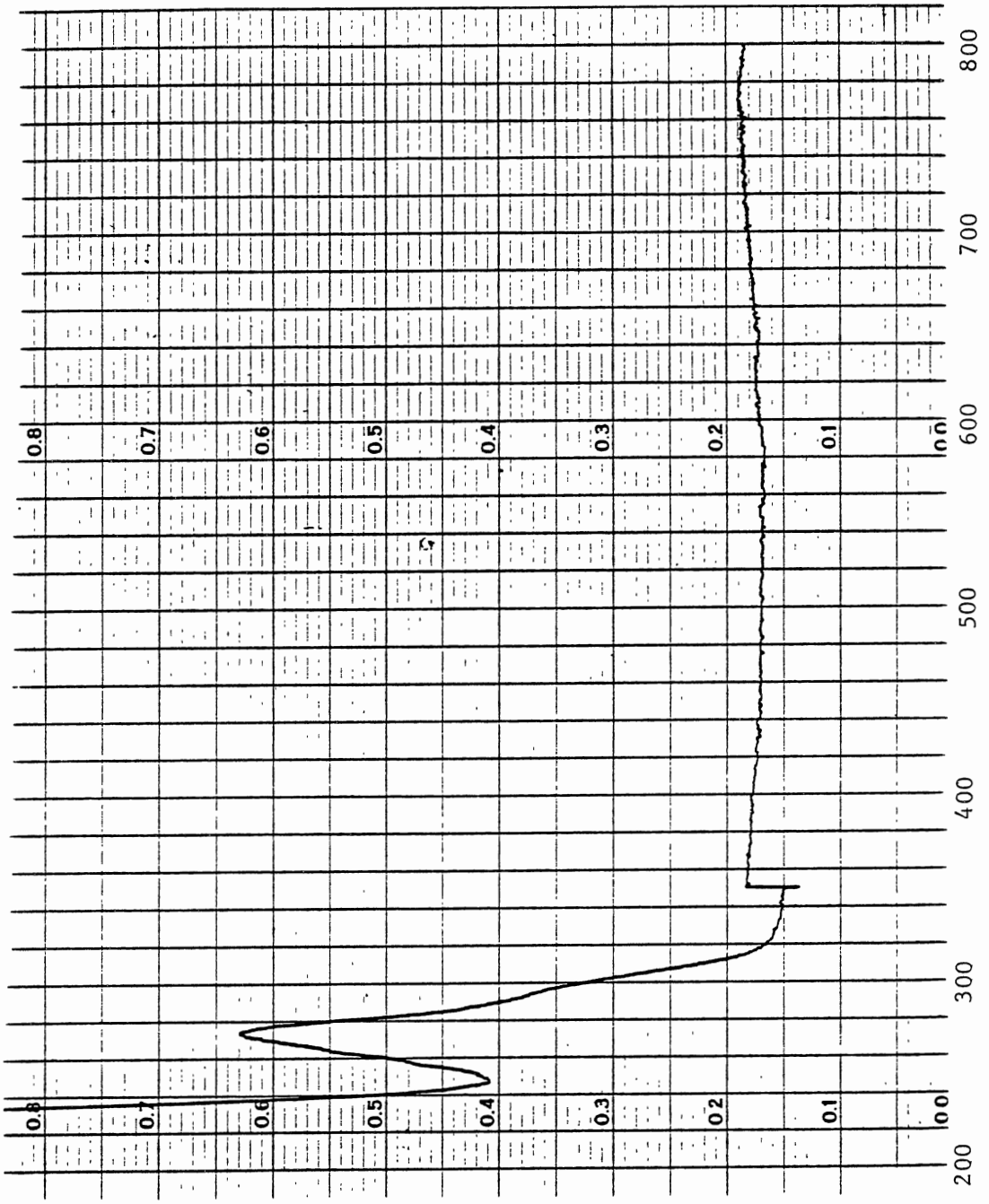

2
1
0·1
0·01
A_{540}
1
5
10
15
20
time (hrs.)

Figure 6. Spectrophotometric scan of a sample of 'M' antibiotic on Cary 14 Spectrophotometer.


0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
00
200
300
400
500
600
700
800

The mass spectrometer showed that it was a structure that contained six atoms of carbon, eight atoms of hydrogen and seven atoms of oxygen ($C_6H_8O_7$: $M^{+\cdot}$192·0270 corresponding to exact mass). It has the following fragmentations as determined by electron impact using the LKB-9000:

$\underline{m}/\underline{z}$ = 156 [$M^{+\cdot}-2H_2O(36)$],

$\underline{m}/\underline{z}$ = 141 [$156-CH_3(15)$],

$\underline{m}/\underline{z}$ = 129 [$M^{+\cdot}-H_2O(18)-COOH(45)$],

$\underline{m}/\underline{z}$ = 112 [$M^{+\cdot}2H_2O(36)-CO_2(44)$],

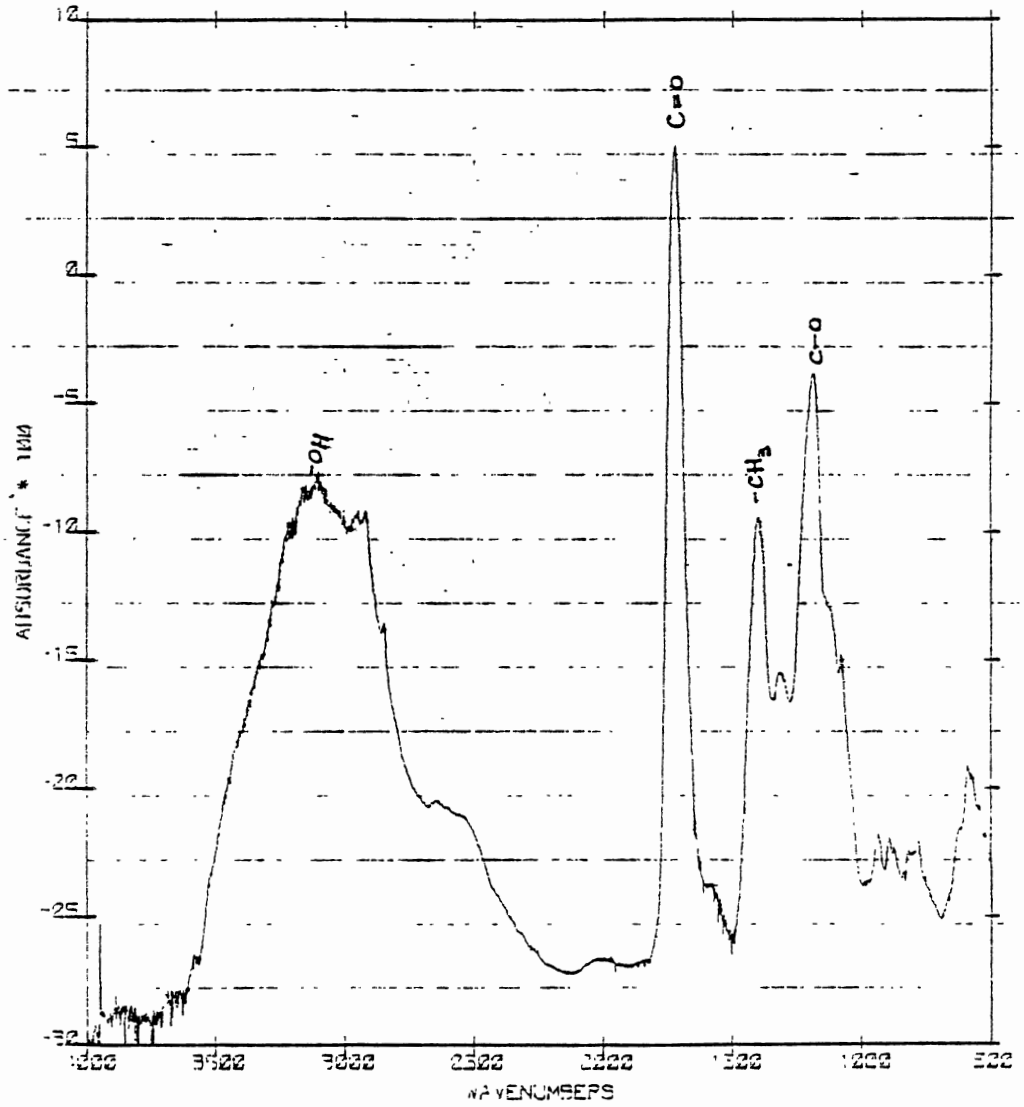
$\underline{m}/\underline{z}$ = 102 [$M^{+\cdot}-H_2O(18)-CO_2(44)-CO(28)$],

and $\underline{m}/\underline{z}$ = 84 [$M^{+\cdot}-2H_2O(36)-CO_2(44)-CO(28)$].

The presence of the functional groups in this fragmentation pattern was further confirmed using infra-red spectroscopy and nuclear magnetic resonance spectroscopy.

Figure 7 shows the infra-red spectrum of the antibiotic. There are four distinct peaks that are distinguishable on this spectrum. A rather broad peak occurred between 2500 and 3500 cm^{-1}. This peak was interpreted as being due to the O-H groups of carboxylic acid. The most intense peak of the spectrum was at about 1725 cm^{-1} which was interpreted as a carbonyl ($\overset{\prime}{C}=O$) peak. A peak of medium intensity occurred at 1400 cm^{-1} which was interpreted as due to a methyl group ($-CH_3$). Another peak also of medium intensity occurred close to 1200 cm. This peak was a little more intense than the $-CH_3$ peak and it was interpreted as a $\overset{\prime}{C}-O$ peak which may be due to carboxylic acids, anhydrides, alcohols, ethers and esters. In this case it was probably due to a carboxylic acid because other identified peaks seemed to point in this direction.

Figure 7. Infra-red spectrum of 'M' antibiotic.



SAMPLE CAST FROM ACETONE . DRIED IN VAC. 10 MIN

SFL=GRUCA2
NSCANS=100
PLM=A

REG=2 DP

RFL=BURYRYM01
NSCANS=100
7/21/83 12:58:19

The ^{13}C NMR spectrum is shown in Figure 8. Carbonyl carbons are strongly deshielded, presumably because of the $\overset{|}{C}{=}O$ bond polarization and so they occur down field. Therefore, peak numbers 8 and 18 which are at about 179 and 176 ppm respectively are carbonyl peaks. The most intense peak in the field (#27) occurred at about 45 ppm. This was interpreted as a methyl peak. A low intensity peak (#20) at about 76 ppm was probably due to a quarternary carbon atom on the molecule. Peak numbers 23, 24 and 32 could not be interpreted with an appreciable level of certainty. However, peak #24 may be due to the solvent.

Based on these spectrophotometric analyses, along with the 'H NMR spectrum in Figure 9, the two chemical structures shown in Figure 10 have been proposed for the 'M' antibiotic.

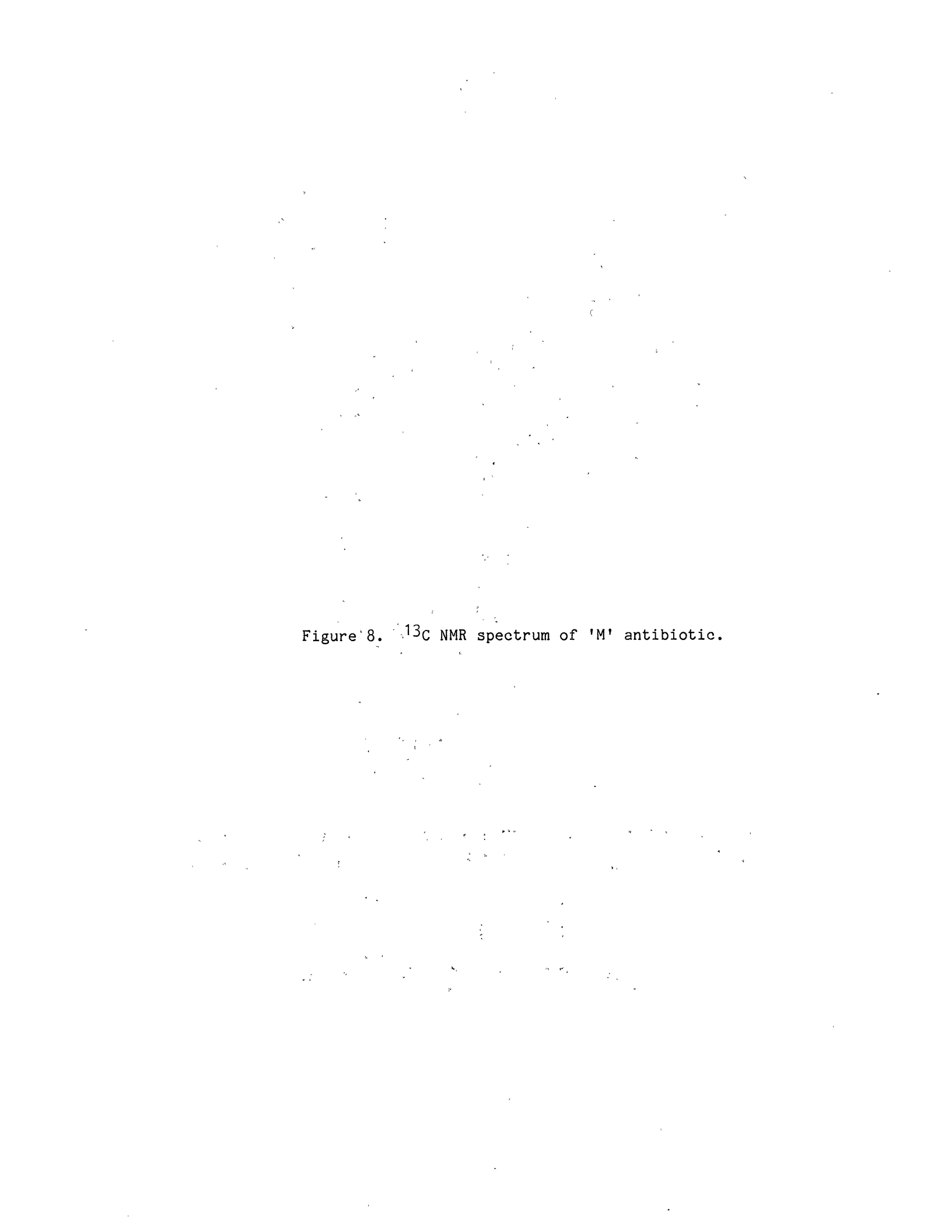


Figure 8. ^{13}C NMR spectrum of 'M' antibiotic.

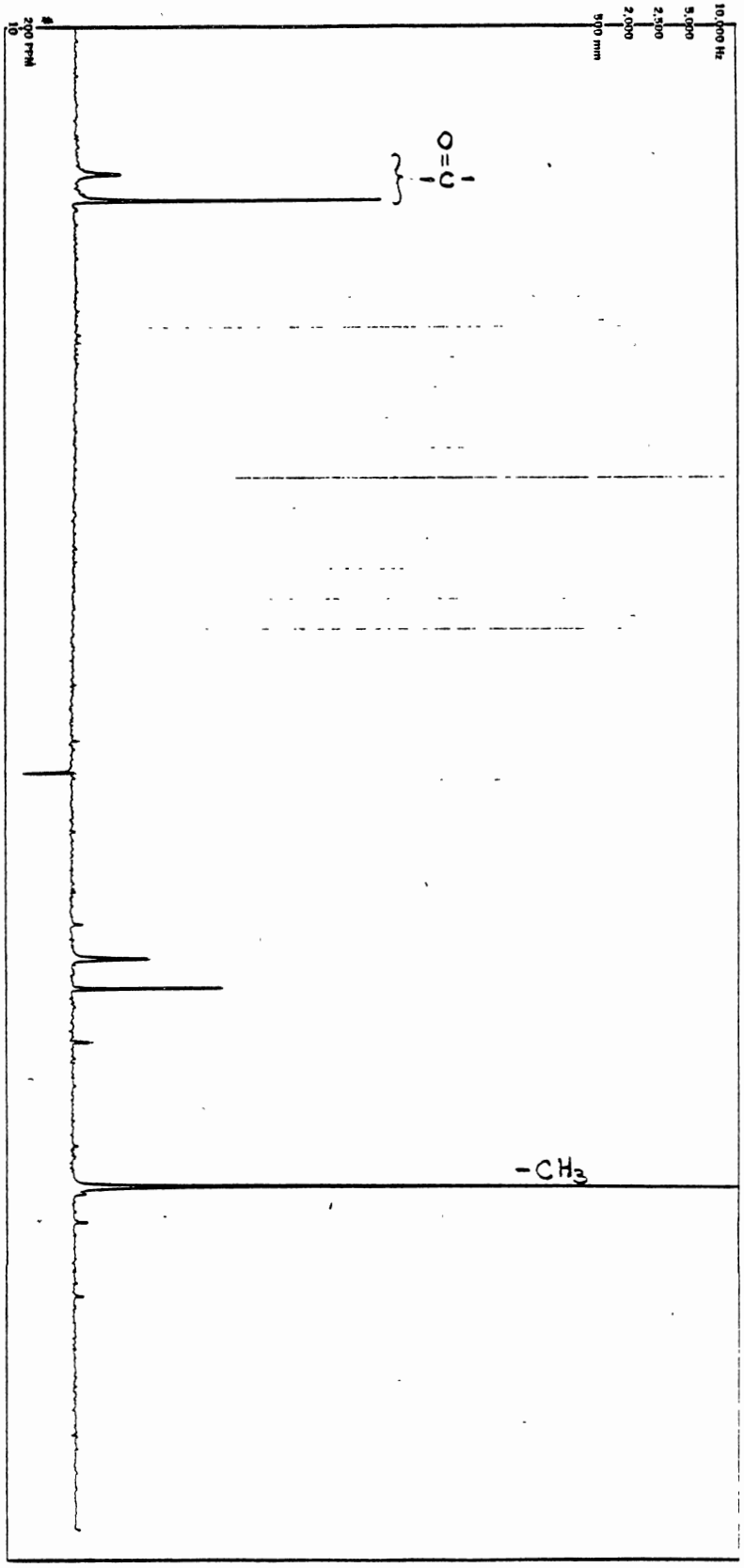

10,000 Hz
5,000
2,500
2,000
500 mm
0
200 PPM
10
O=C
-CH3

Figure 9. ^{1}H NMR spectrum of 'M' antibiotic.

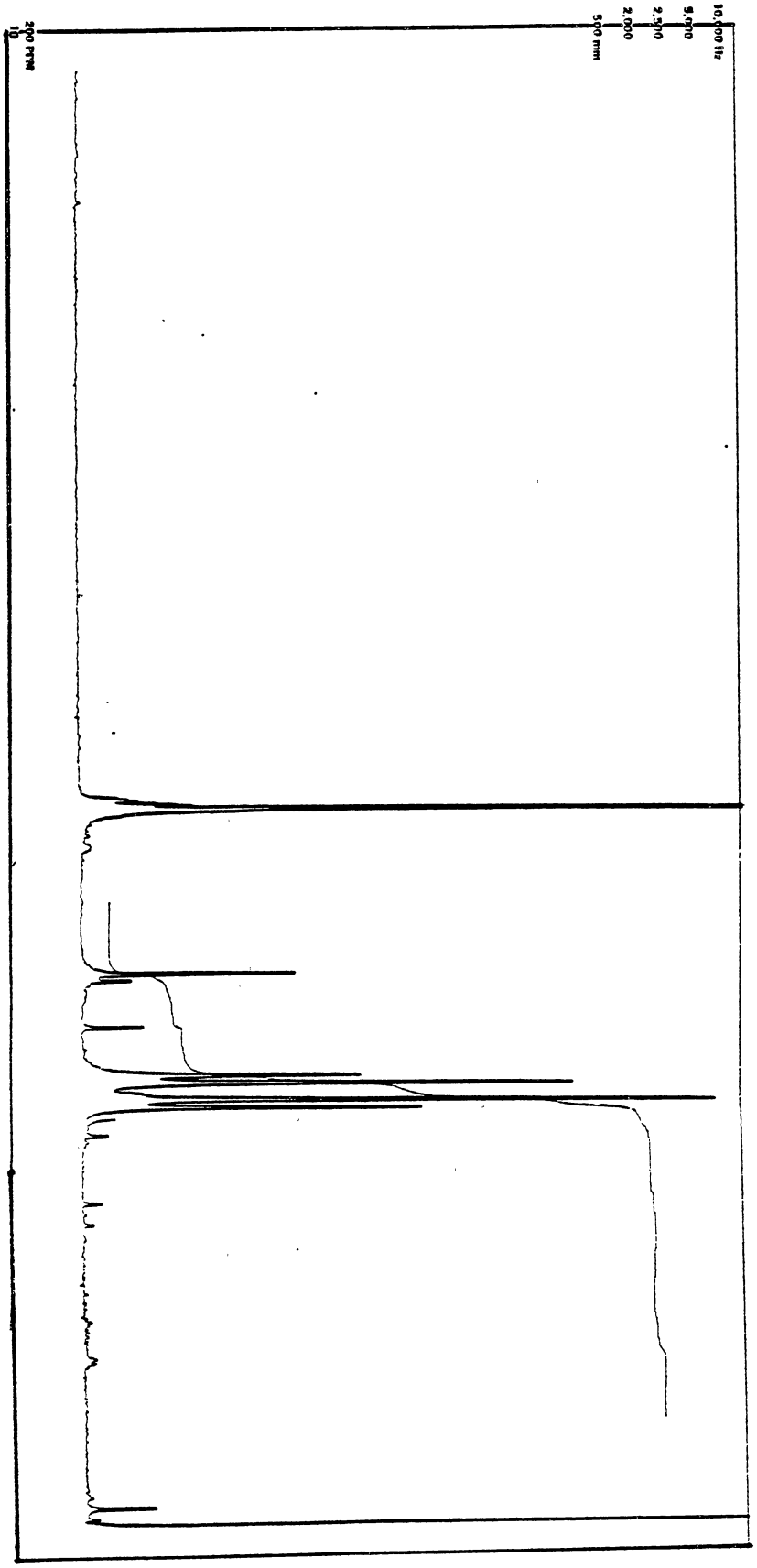
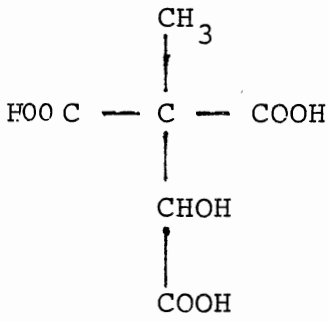
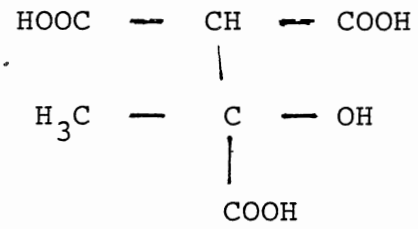

10.1000 Hz
5.0000
2.5000
2.0000
500 mm
200 PPM
10

Figure 10. Proposed chemical structures for 'M' antibiotic.



1-Hydroxy-1,2,3
Propane Tricarboxylic
Acid

or



2-Hydroxy-1,1,2
Propane Tricarboxylic
Acid

CHAPTER IV

DISCUSSION

The search for ideal chemotherapeutic agents that act as "magic bullets", selectively killing pathogenic organisms while sparing their hosts, probably dates back to Paul Ehrlich in Germany in the latter part of the nineteenth century. The term "antibiotic" was coined by Waksman in 1942. It was broadly defined as any product of microbial metabolism which is capable of inhibiting the growth of other microorganisms or even killing them in relatively very low concentrations. With the advent of the second world war, the need for antibiotics became more necessary than ever. Driven by this urgency the "Oxford team" of Chain, Abraham and Florey, and scientists at the American Northern Regional Research Laboratories in Peoria, Illinois, in 1939, purified penicillin, whose activity was first demonstrated by Alexander Fleming ten years earlier. Since then, about 3,000 antibiotics have been catalogued, most of them, however, have not found chemotherapeutic use. Perlman (1974) listed 91 antibiotics that are commercially produced by microbiological processes and are, for the most part, used in chemotheraphy.

Often, the discovery of a new antibiotic is an accidental occurrence and the 'M' antibiotic investigated in the present work is no exception. As stated earlier in the introduction, the activity of the 'M' antibiotic was first observed by E.A. Grula when zones of inhibition of certain bacteria were noticed in areas contiguous with the growth of

B. bassiana strain 7. Subsequent work revealed two zones of inhibition distinguishable on paper chromatograms. The 'M' antibiotic corresponded with the zone which occurred about half way up the chromatogram. After the initial characteristics were checked against those of other antimicrobial compounds produced by B. bassiana, it was decided that the 'M' antibiotic was different from them and further research was initiated to produce and purify it. Champlin (1981), has done some initial work on production and purification of the antibiotic.

In the present work, initial purification of the 'M' antibiotic was achieved using solvent extraction partitioning. It was determined that acetone would precipitate some components of the GAC spent medium at a pH of about 3.0, while the 'M' antibiotic remained in the supernatant. At this point, it was hypothesized that the antibiotic molecule had some protonatable group (s) which upon protonation at low pH (3.0) made it more soluble in organic solvents than it was without protonation. As earlier shown in the purification chart in Chapter II, it took five times the volume of the spent medium of acetone to achieve the optimum partitioning. Apparently, this acetone-water ratio was non-polar enough to precipitate the more polar components of the medium and retain the 'M' antibiotic in the supernatant. Also, extracellular proteins in the medium would have been precipitated.

The next step in the solvent extraction procedure involved the extraction of the antibiotic from the supernatant of the acetone precipitation into ten volumes of isoamyl alcohol. With protonation using 1 N HCl, the antibiotic became relatively soluble in isoamyl alcohol. Based on the activities obtained from the extracts with disc assay and chromatographic assay, it was estimated that about ten percent

of the antibiotic was extracted from the spent medium using isoamyl alcohol. The isoamyl alcohol extract was either dried by air-blowing with an air stream, or it was re-extracted into glass-distilled water (one-tenth the volume of the isoamyl alcohol). The extract was then frozen overnight at -20°C and lyophilized. The antibiotic was found to move from isoamyl alcohol back into the water without raising the pH from the initial 3.0 because the antibiotic was only sparingly soluble in isoamyl alcohol even at the low pH. It required 100 ml of isoamyl alcohol to extract an estimated ten per cent of the antibiotic from 10 ml of spent medium. The dried isoamyl alcohol extract was freely soluble in water. The isoamyl alcohol extraction step is similar to some steps in enzyme purification procedures in which a high level of purification is accompanied by a low product yield.

The product obtained from the combined acetone precipitation and isoamyl extraction was considered pure enough for use in most of the experiments. Evidence for this was obtained mainly from chromatography followed by bioautography in which there was no tailing or heading in the inhibition zone produced by the extract.

Further purification of the acetone supernatant was also done by gel filtration using Sephadex G-25-150 beads with an exclusion limit of 100 to 5,000 daltons. However, it was found that this method did not provide a better alternative to the isoamyl alcohol extraction method. It appeared that the 'M' antibiotic was not adequately resolved from other components of the spent medium. This method of purification is based on molecular size and shape.

Ion exchange chromatography was another method used in the purification of the 'M' antibiotic. This method was based on the

assumption that the 'M' antibiotic molecule had some protonatable and therefore ionizable group(s) which made it possible for it to bind to either cationic or anionic exchange resin at certain pH values and be dissociated from either when the pH was appropriately changed.

Once the molecular composition of the 'M' antibiotic had been established, its properties became easier to explain based on three carboxyl groups present on the molecule which could ionize.

The acid activation property could be explained on the basis of the protonation of the carboxyl groups at low pH resulting in the neutralization of the negative charge on the molecule. Since charged molecules can only traverse the cell membrance with difficulty, it was easier for the fully protonated molecule to get into the cell. However, when the pH was adjusted back to about 7.0, the molecule lost a part of its protons and became negatively charged again and once again had difficulty traversing the cell membrane. Once inside the cell, the differences in the amount of inhibition obtained with different bacteria is probably a question of susceptibility. This, however, needs to be examined further, to determine the molecular basis of antibiosis and susceptibility.

It was determined by Champlin (1981) that citrate was the best precursor of the antibiotic of all the tricarboxylic acid intermediates tested. It was also determined that more 'M' antibiotic was produced when the amount of citrate in the medium was increased. Radioactive labeling has been employed in the present work to confirm that citrate was the best precursor of the 'M' antibiotic. It would appear that the synthesis of the 'M' antibiotic is due to a pathway that branches off the tricarboxylic acid cycle in which citrate is somehow modified into

the antibiotic. This is probably why its synthesis is dependent on the amount of citrate available. The antibiotic is similar enough to citrate to probably act as a competitive inhibitor of the enzyme aconitase that converts citrate to isocitrate in the tricarboxylic acid cycle, thus shutting off the cycle. The compound thus probably acts as a metabolic inhibitor.

A new broad spectrum antimicrobial compound was isolated and purified from the spent medium of a strain of Beauveria bassiana. This compound was found to be activated when the pH was lowered and its chemical structure was determined by mass, nuclear magnetic resonance, and infrared spectrophotometry. It was found to be a tricarboxylic acid similar to citric acid (Appendix). A search through the existing literature revealed no previous mention or characterization of this compound and so it is presumed to be a new compound. It was given the trivial name of "GRULYSIN" in honor of E.A. Grula who first noticed its activity.

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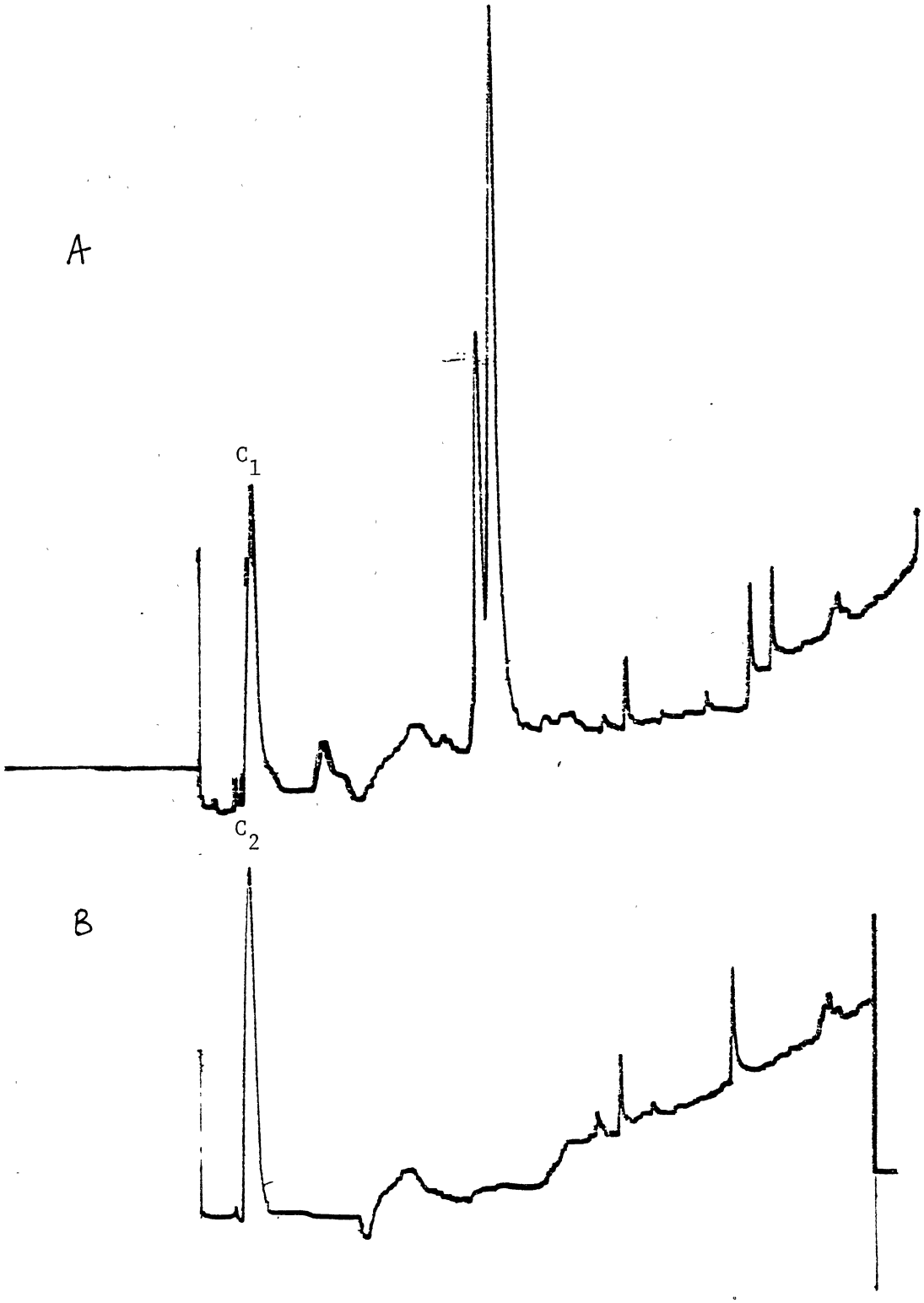
High Performance Liquid Chromatography (HPLC)

A sample of the 'compound', 'M', was subsequently analyzed using the HPLC. One hundred µl of a solution of 1 mg/ml in the sample was injected into a Model 2-2919 sample injection valve form Supelco Co., Bellefonte, Pa., and pumped by a Model 6000 chromatographic pump from Waters Associates Co., Medford, Ma., into an Ultrasphere ODS reversed phase column (250 x 4.6 mm I.D.) obtained from Rainin Instrument Co., Woburn, Ma. The effluent was monitored by a UV detector from Waters Associates, Model 440 (280 nm) and another detector from Isco, Lincoln, Ne., Model 82133 (214 nm) and recorded on a 280 nm chart recorder from Sargent-Welch, Baton Rouge, La. (0.2 cm/min) and a 214 nm recorder form Isco (0.25 cm/mn) respectively. The column was eluted at room temperature with a linear gradient formed in a custom-made gradient maker. The limiting solvent chamber contained 30 ml of 90% methanol (J. T. Baker Chemical Co., Phillipsburg, N. J., HPLC Grade), and the mixing chamber contained 0.1% trifluoroacetic acid (TFA) (Liao et al., 1982). The flow rate was 1 ml/mm. After each chromatographic run, the column was washed with 20 ml of methanol.

A comparison of the sample profile with that of citrate profile at 280 nm (Figure 11) shows the presence of a peak (C_1) on the sample spectrum which corresponds with the strongest peak (C_2) on the citrate spectrum. This was interpreted as meaning that the sample was related to citrate.

Figure 11. High performance liquid chromatographic profile of 'M' antibiotic (A) compared with that of citrate (B).


A
C_1
C_2
B

VITA[2]

Akinlolu Adebayo Adefarati

Candidate for the Degree of

Master of Science

Thesis: PURIFICATION, CHEMICAL COMPOSITION AND PRIMARY MECHANISM OF ACTION OF AN EXTRACELLULAR ANTIMICROBIAL COMPOUND PRODUCED BY BEAUVERIA BASSIANA

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